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**How different host genotypes alter the virulence-
transmission trade-off in *Drosophila melanogaster*-
Pseudomonas entomophila complex?**

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ABSTRACT

Parasitism is seemingly the most common biological interaction that can be established between two organisms. Its importance and interest are not limited to the biological sciences field, but also to agriculture, livestock and human and veterinary medicine. Hence, the study of the interactions between parasites and host is necessary to comprehend and better fight infectious diseases. In these relations, a parasite takes advantage of the host by consuming its resources for its own benefit, such as reproduction and transmission to other hosts. By exploring host resources the parasite causes damage, which is usually called virulence.

The trade-off theory states that virulence is an unavoidable consequence of parasite transmission. Hence, a parasite cannot increase its transmission indefinitely because it causes increases damage to the host, which increases the probability of killing the host that compromises transmission. Studies that show this apparently simple constraint are scarce and few have explored the influence of the host genetic background in this model.

In this study, we make use of the pathosystem composed by *Drosophila melanogaster* and its natural pathogen *Pseudomonas entomophila*, through the natural oral route of infection, to test for a trade-off between these traits during infection. Moreover, we aimed to study how different host genetic backgrounds influence each trait and identify its putatively causative genes.

First, we established a protocol to accurately measure virulence and transmission in this system using an *D. melanogaster* outbred population. We verify a positive correlation between virulence and transmission. Second, we apply this protocol to different host genetic background. As in the outbred population, we find a positive correlation between traits. Moreover, we see that the host genetic background greatly influences the infection traits. However, our data do not fully corroborate the occurrence of a trade-off.

Additionally, we study the genetic bases that underlie the different susceptibilities to infection. Surprisingly, our results do not evidence any candidate gene related with immunity or gut development.

Our results are still inconclusive and a complete dataset is required to fully address the questions we started off with. However, with this work, we have established an experimental system for the study of the correlation between virulence and transmission between *D. melanogaster* and *P. entomophila*. Moreover, our preliminary data showed that even in laboratory established systems there is still much to discover. Expectantly, future studies will address and answer questions that are in the basis of this project and that emerged from obtained results.

Key words: Parasitism; Trade-off virulence-transmission; *Drosophila melanogaster*; *Pseudomonas entomophila*.

RESUMO

Parasitismo é uma interação que envolve dois organismos, um parasita, que retira vantagens da interação às custas do hospedeiro. Mais precisamente, o parasita utiliza os recursos do hospedeiro para seu proveito, sobretudo para se reproduzir e ser transmitido. Nesta interação o parasita devido à exploração dos recursos causa dano ao hospedeiro, ao que habitualmente se chama virulência. A virulência de um hospedeiro pode ser considerada uma característica que comprometa a interação e, assim, desvantajosa para o parasita. Seria, então, de esperar que houvesse uma seleção de parasitas que não causassem dano no seu hospedeiro. No entanto, se esta característica desvantajosa estiver correlacionada com uma característica vantajosa para o parasita, melhor se explicaria a existência de virulência em relações parasíticas. É a partir desta hipótese que surge a teoria do *trade-off*.

A teoria do *trade-off* entre virulência e transmissão foi desenvolvido por Anderson e May, que no seu estudo correlacionam pela primeira vez o efeito do parasita no tempo de recuperação do hospedeiro. Esta hipótese defende a existência de uma relação de constrangimento entre as características de virulência e transmissão. Mais precisamente, que a virulência não pode aumentar indefinidamente sem comprometer a transmissão. O início da curva de *trade-off* é caracterizada por uma correlação positiva entre as duas características, em que o custo de aumentar a exploração dos recursos do hospedeiro, logo, a virulência, é acompanhado pelo aumento de transmissão. No entanto, esta curva satura, atingindo o valor ótimo, em que a transmissão é maximizada. A partir deste ponto o aumento da exploração de recursos leva a um aumento de virulência e a uma redução da transmissão, devido ao excesso de dano no hospedeiro pelo parasita, levando a que os hospedeiros morram antes do parasita ter oportunidade de se transmitir.

Apesar da simplicidade da hipótese de *trade-off*, estudos que demonstrem esta correlação são escassos. O primeiro caso de estudo foi desenvolvido nas décadas de 50 e 60 por Fenner e colegas. Os resultados destes estudos sobre o vírus de mixomatose em populações naturais de coelhos europeus (*Oryctolagus cuniculus*), foram utilizados para fundamentar a hipótese do *trade-off*. Atualmente, novos estudos têm caracterizado a correlação entre virulência e transmissão em diversos sistemas, estudando a influência de diferentes estirpes de vírus e da interação com organismos vetores na infecção de hospedeiros.

No presente trabalho, de modo a testar a teoria do *trade-off* entre transmissão e virulência, caracterizámos esta correlação numa população geneticamente variável de *Drosophila melanogaster*. Como patogénico, recorremos a uma estirpe de bactéria que infecta populações naturais do nosso organismo, *Pseudomonas entomophila*. Os principais objetivos da tese são:

- i. Desenvolver um novo protocolo que permita a medição de virulência e transmissão de um patógeno numa população *outbred* (com variabilidade genética) de *D. melanogaster*. E deste modo, caracterizar a relação entre as duas características no nosso complexo modelo.
- ii. Estudar o efeito de diferentes *backgrounds* genéticos do hospedeiro nas medições de virulência e transmissão.
- iii. Investigar as bases genéticas subjacentes aos fenótipos observados.

Para o novo protocolo, adaptámos um protocolo estabelecido e anteriormente utilizado do laboratório, que apenas nos permitia medir virulência, de modo a medir as duas características na mesma população e geração. De modo a distinguir entre indivíduos utilizados para medir virulência e indivíduos para medir transmissão, recorremos a uma segunda população com um fenótipo facilmente distinguível da primeira.

Estudamos a quantidade de bactéria presente em indivíduos infectados e caracterizamos a dinâmica da bactéria nas primeiras 72 horas após infecção. Observamos um decaimento constante da quantidade na população e o declínio do número de indivíduos infectados. Estes resultados vão de encontro a resultados anteriores que afirmam que a rápida eliminação da bactéria do sistema digestivo do hospedeiro contribui para a diminuição da mortalidade na população. Apesar da tendência de redução em toda a população, observamos a persistência de valores intermédios da quantidade de bactéria numa porção da população infectada (10%) nas últimas horas analisadas. Estes resultados parecem indicar a existência de tolerância no nosso sistema, visto que neste período a taxa de mortalidade é bastante reduzida.

Devido a constrangimentos temporais, ao medir virulência e transmissão na população *outbred*, apenas medimos valores de virulência na parte inicial do espectro (Virulência: 0.5-38%; Transmissão: 3.125-66.67%). Observamos no nosso sistema uma correlação positiva entre as duas características, o que vai de encontro ao que é proposto na hipótese de *trade-off*, referente à parte inicial da curva, e a resultados de estudos anteriores. No entanto, os nossos resultados não demonstram uma saturação da curva, igualmente teorizado. Isto deve-se, provavelmente, à falta de mais informação, nomeadamente níveis de virulência mais elevados que, teoricamente, comecem a comprometer a transmissão. Uma outra hipótese é que a correlação entre virulência e transmissão no nosso sistema não revele um *trade-off*.

De modo a testar o efeito de diferentes backgrounds genéticos do hospedeiro nas características de uma infecção, recorremos às linhas DGRP (*Drosophila* Genetic Reference Panel). A população DGRP consiste em mais de 200 linhas *inbred* (com reduzida diversidade genética) derivadas de uma população natural (Raleigh, USA). Este painel permite o estudo do background genético para determinado fenótipo. Devido a todas as linhas estarem sequenciadas, este painel consiste numa livraria viva de polimorfismos, o que permite a realização de Genome Wide Association Studies (GWAS), de modo a identificar as bases genéticas de fenótipos observados.

Utilizando as populações DGRP como dadores de bactéria para uma segunda população, medimos virulência em 92 linhas do painel. Obtivemos percentagens de mortalidade ao longo de todo o espectro (2,1-98%). Confirmamos, que há influência da genética do hospedeiro na suscetibilidade a infecção.

Para o fenótipo de transmissão, devido a constrangimentos temporais, apenas 7 linhas foram medidas em. Obtivemos medições de virulência e transmissão ao longo de ambos os espectros (2,1-87% e 3-87,5%). Apesar do reduzido número de pontos, observa-se uma correlação positiva entre as duas características. No entanto, este resultado tem de ser visto com cuidado devido ao reduzido número de pontos obtidos para caracterizar a correlação entre as características.

Utilizando os dados fenotípicos, realizamos o GWAS para o fenótipo de virulência. Obtiveram-se cerca de 26 SNPs significativos. No entanto, a maioria dos resultados centra-se em genes com função desconhecida e os restantes, não aparentam qualquer relação com imunologia ou alterações no sistema digestivo. A única exceção é o gene *hs6t* que está relacionado com o desenvolvimento da traqueia.

Os principais resultados deste estudo têm de ser vistos com cuidado devido ao reduzido número de pontos utilizados para construir as duas correlações entre características. No que refere às curvas de correlação entre características, observa-se uma correlação positiva em ambas populações testadas. A falta de mais dados, quer de maior virulência, na população *outbred*, quer de um maior número de linhas testadas, no estudo usando DGRPs, pode ser apontada como a principal causa de ausência de curva de saturação. Uma outra causa para a ausência de uma curva no nosso sistema, pode dever-se a que o nosso sistema parasita-hospedeiro, seja caracterizado por um outro modelo que não uma curva *trade-off*. No que se refere aos estudos de bases genéticas, não se observaram resultados expectáveis. A ausência de resultados concludentes pode dever-se ao novo protocolo ou ao reduzido número de linhas testado. No

entanto, este projeto permitiu o estabelecimento de um novo protocolo que permite a medição de transmissão utilizando o organismo modelo *D. melanogaster*, embora ainda precise de ser otimizado, constituindo uma ótima ferramenta para ajudar a compreender melhor a relação complexa que se estabelece entre hospedeiro e parasita num sistema controlado.

Palavras chave: Parasitismo; *Trade-off* virulência-transmissão; *Drosophila melanogaster*; *Pseudomonas entomophila*; Genome Wide Association Study.

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LIST OF ABBREVIATIONS

CFU	Colony forming unit
DEL	Deletion
DGRP	<i>Drosophila</i> genetic reference panel
dpi	Days post infection
GWAS	Genome wide association study
INS	Insertion
OD	Optical density
SNP	Single nucleotide polymorphism

1. INTRODUCTION

1.1. Parasitism: virulence, transmission and the trade-off

All organisms are in constant contact with other organisms whether they are from the same or from another species. Among these biotic interactions there is a specific set that is characterized by being long-term interactions between two organisms. These interactions are called symbiotic interactions¹. There is a spectrum of classifications that can be attributed to a symbiotic association², but the vast majority seems to fall into one of the following categories: mutualism, commensalism and parasitism³. Parasitism is seemingly the most common biological interaction in the animal kingdom^{4,5} and describes associations between organisms where one benefits at the expense of the other.

Some authors have argued that parasitism is the selective force behind some of the most important theories explaining the genetic diversity found in natural populations⁶⁻¹⁰ and the evolution and maintenance of sexual reproduction^{7,11,12}. Because of its biological, medical and economic importance, parasitism is one of the most studied interactions^{13,14}. The connection between parasite and host is beneficial to the first but adverse to the second. The pathogen uses host resources for its own advantage, to reproduce and transmit. The harm that is done to the host is defined as parasite virulence, which ultimately can lead to the death of the host¹⁵⁻¹⁷.

From the parasite perspective the most favourable outcome is high reproduction rate and consequently increase the probability of transmission⁶. To this objective, the parasite increases exploitation of host resources¹⁸, which leads to an rise in the parasite fitness. However, the increase exploitation of resources has as consequence an increase in harm to the host. This harm can be so severe as to cause the death of the host. In most cases, this leads to the compromise of parasite transmission, hence, to a decrease in host fitness since the number of new infections will be reduced due to the host dying too quickly¹⁹.

There is thus a constraint between virulence and transmission during a parasitic interaction. This constraint is at the core of the Virulence-Transmission Trade-off hypothesis (Figure 1.1), a hypothesis first developed by Anderson and May (1982)⁶ and complemented by Ewald (1983)¹⁸. This hypothesis was mostly based on the results from studies developed by Fenner^{6,18,20,21}. They studied, through several years, the evolution of the myxoma virus in natural populations of European rabbits (*Oryctolagus cuniculus*) and in different regions of the world. In these studies, they observed that a single extremely virulent strain (mortality>99%) when released into rabbit populations in Australia and Britain in the early 1950s, but over the next decade evolved to several different strains that have dissimilar stable levels of virulence²⁰⁻²². At a later stage, they observed that this affects the number of vectors, mosquitos and flies, which become infected and transmit the pathogen to susceptible hosts²³. This system still represents the most complete study that correlates virulence and transmission during infection, as they observe a correlation between the harm done to the host, the time it takes to recover from infection and the number of infected vectors (i.e. transmission)²⁴.

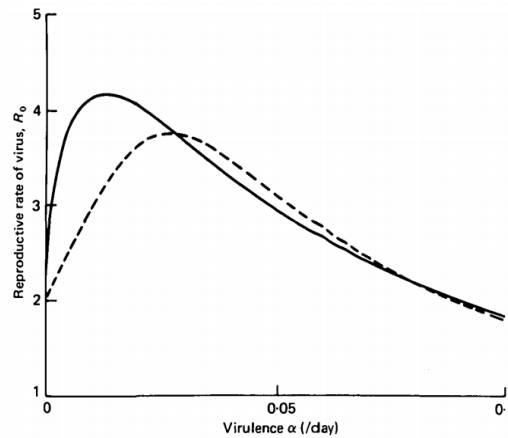


Figure 1.1. Trade-off curve. Taken from Anderson and May (1982)⁶. Relation between intrinsic reproductive rate, R_0 , and virulence, α . The R_0 reaches a maximum value for an intermediate grade of virulence. The R_0 can be seen has a proxy to transmission rate. Too high α kills off hosts too fast, diminishing the capacity to transmit the infection; but too low α corresponds again to weak transmission. Both lines correspond to empirical data.

This theoretical hypothesis postulates that there is an intermediate optimum point until which the transmission rate increases more rapidly than the cost. At the intermediate optimum value, the transmission is maximized and the parasite attain the most profitable deal. After that optimal point, increasing transmission is exceeded by the rise in the cost, meaning that the parasite is causing increased damage but less transmission. Indeed, since the harm is so great, it kills the host before the pathogen has the opportunity to infect other hosts, resulting in the decrease of transmission probability (Figure 1.1)^{6,24-26}.

Despite the simplicity of this hypothesis, empirical data to support it is actually scarce. Recent studies have obtained data that corroborate this hypothesis²⁴, others not²⁴. These studies use different hosts and vectors to analyse how virulence and transmission are correlated. Here we analyse the advantages and disadvantages of the main studies with empirical data that supports the trade-off hypothesis.

In the study made by Jensen and colleagues (2006)²⁷, they used a less complex system where a bacterium (*Pasteuria ramose*) is used to infect *Daphnia magna*. They study the correlation between the longevity of the host and the number of spores produced by the parasite. They observe a high variation in the time the bacteria takes to kill the host. Moreover, bacteria that kill hosts early or later, produce less spores when compared with intermediate level of virulence. These results suggest an optimum intermediate level of virulence for this system, when the transmission is maximized, which is postulated by the trade-off²⁷. Hence, this study shows a positive correlation between traits in the initial part of the model, followed by a saturation of the curve at intermediate levels of virulence and then a negative correlation in the final portion of the curve. However, in this system the spores are not released until after host death, which means that contrary to what is postulated by the trade-off hypothesis, host death does not compromise transmission.

An empirical test of the virulence-transmission trade-off was performed by de Roode (2008), in which they use several protozoan virus strains, *Ophryocystis elektroscirrha*, to infect monarch butterflies (*Danaus plexippus*)²⁸. They demonstrated the existence of a trade-off which is caused in part by the fact that transmission increases slower than linearly with the number of spores emitted from an infected insect (Figure 1.2). However, they measure transmission by quantifying the number of spores in eggs laid by infected mated females. Hence, this tests measured predominately the importance of vertical transmission instead of horizontal transmission like is postulated by the trade-off.

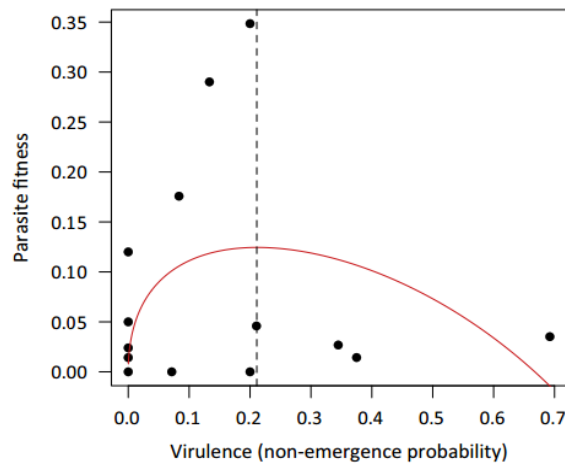


Figure 1.2. Trade-off virulence-transmission. Taken from Alizon and Michalakakis²⁵. Graphical representation of the trade-off hypothesis using data obtained in the study of de Roode (2008)²⁸.

More recently, Doumayrou and colleagues (2013)²⁹ tested for the first time the existence of trade-off on a plant pathogen. They infected *Brassica rapa* plants with nine natural isolates of *Cauliflower mosaic virus*. They estimated virulence on the individual host plants and for transmission they used the aphid vector *Myzus persicae*. As predicted by the trade-off hypothesis, they observed a positive correlation between traits at low virulence levels until an intermediate level of virulence where they observe the highest level of transmission. Although the data indicates a saturating of the curve, there is not a complete reversal in the correlation between the traits. This may be due to higher virulence levels, that would possibly compromise transmission, not being represented in this study.

Even though some studies have used systems that diverged from that used to established the trade-off hypothesis (see more in²⁴), they still obtained similar correlation between traits, which may show some flexibility of this model. Still, due to the difficulty to measure complex traits like virulence and transmission, empirical data that clearly demonstrate the trade-off are scarce²⁵. Due to these factors there have been an increase challenge to this hypothesis^{25,30}.

One factor that may affect infection is the host genotype³¹. Indeed, for the pathogen's not all hosts are equal, hence including the genetic variability in studies will greatly increase the relevance of results obtained³². It has been demonstrated that host heterogeneity influences infections traits such as virulence^{9,33}, reservoir potential (i.e. specie that is infected and serves as a source of infection for another specie)³⁴ or transmission^{35,36}. Particularly, the studies of Magwire (2012)⁹ and Bou Sleiman (2015)³³ that tested the outcome of infection with single strains of pathogen, virus and bacteria respectively, in different host genetic backgrounds, demonstrates the importance that host heterogeneity has to infection. They observed a strong influence of the host genotype in the susceptibilities to infection with either parasite. These results support the idea that the pathogen virulence is the admixture of several susceptibilities of the host.

Here, we aim to test the correlation between virulence and transmission in a *Drosophila melanogaster* pathogen, for the first time. Furthermore, instead of virus, that is the most commonly used in studies addressing the trade-off hypothesis^{20,21,28,29,37}, we used a strain of a non-vector dependent bacterium, *Pseudomonas entomophila*. Therefore, we aim to characterize the interaction between virulence and transmission in our less complex system (without a vector organism), like in the study of Jensen (2006)²⁷, and, this way open the path for more complex studies. Thus, we hope to use the tools available for our model system to shed some light on this specific system and more fundamentally, to better understand parasite biology.

1.2. Study System

1.2.1. The host: *Drosophila melanogaster*

Drosophila melanogaster, commonly known as fruit fly or vinegar fly, is a model system widely used in biological research, including evolutionary and developmental biology. Because of its widespread use across many research areas, the model system *D. melanogaster* is ideal to address questions related to parasite infection. Specifically, because it is a model system for invertebrate immunity³⁸ and given the vast genetic tools available³⁹, *D. melanogaster* stands out as a powerful model for the study of host-pathogen interactions^{13,38,40,41}.

Recently there have been studies that unravel the evolution of *D. melanogaster* responses to pathogens¹³ and how different infection routes impact on host evolution¹³. Despite the greater amount of systemic infection studies (see more in¹³), injuries are probably not the most frequent source of pathogen entry *D. melanogaster*, the lead role being played by ingestion^{13,38,42}.

The *Drosophila melanogaster* Genetic Reference Panel (DGRP)^{39,43} lines are an excellent tool that allows to decompose and analyse the genetic variability of a natural population. It consists of around 200 fully sequenced lines in which all individuals in each line are genetically equal and genetically different from all other lines. The combination of all lines is thought to represent the genetic variability of the original natural population from which they were derived.

The capacity to decompose the natural variation of a population and the possibility of study its different constituents individually, has constituted a major step in understanding the genetic bases of phenotypic variation. The DGRPs allows that complex and time consuming Genome Wide Association Studies (GWAS) to be done in a less time-consuming way. Recently, several studies have made use of this tool unravelling the genetic bases of phenotypes like sleep⁴⁴, longevity⁴⁵, growth⁴⁶ and more recently, viral and bacterial infection resistance^{9,33}.

Conclusively, the specific characteristics of these inbred fly lines allows assessment of the impact of infection on distinct but constant genetic backgrounds, in order to tease out the effect of the genotype from that of environmental factors³⁹.

1.2.2. The pathogen: *Pseudomonas entomophila*

Pseudomonas entomophila is a Gram negative bacterium first collected from a fly in Guadeloupe⁴⁷, that is highly pathogenic for *D. melanogaster*, as well as for other insect species^{48,49}. It has been shown that *P. entomophila* infection causes severe irreversible intestinal epithelial damage⁵⁰⁻⁵⁴. Also, it is the only *Pseudomonas* species capable of activating a systemic immune response, mainly through the Imd pathway^{38,40}, in both adult and larvae of *D. melanogaster*⁴⁹. The complete sequencing of this bacterium showed a metabolically versatility which allows the colonization of a large range of habitats, including soil, rhizosphere and aquatic systems⁴⁸. Moreover, the analysed of the genome revealed the existence of large sets of genes encoding putative virulence factors along with regulators that control their expression⁴⁸.

1.2.3. The model complex: *Drosophila melanogaster* - *Pseudomonas entomophila*

As discussed above, it appears likely that the diversity in natural populations affects the infection dynamic between host and parasite. But studies that address the influence of host genetics the host-parasite relation are scarce. One study has associated the susceptibility to viral infection with genetic markers using the DGRP lines⁹. Moreover, recently Bou Sleiman and colleagues³³ looked for differences in molecular and cellular immunocompetence upon *D. melanogaster* oral infection *P. entomophila*.

In this study, 140 DGRP lines were subjected to oral infection with *P. entomophila* (Figure 1.3)⁴⁷. They found a striking variation in survival, hence concluding for considerable genetic variation in susceptibility to enteric infection. From the GWAS results they conclude that differences in susceptibility to infection are probably due to multiple loci with relatively small effects. Further studies trying to characterize this associations might help us to understand better this complex interaction between pathogen and host.

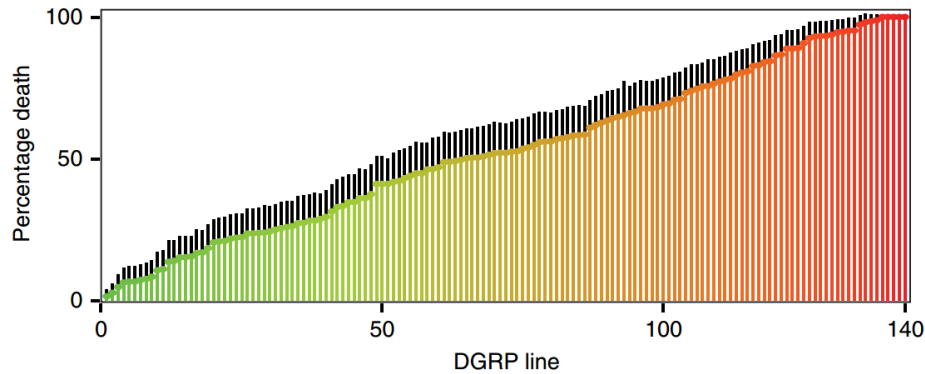


Figure 1.3. Variability of susceptibility to oral infection with *P. entomophila*. Taken from Bou Sleiman (2015)³³. Measurement of fly survival 3 days after oral infection with *P. entomophila*.

1.3. Objectives

Theory predicts a transmission-virulence trade-off in a host-parasite interaction. Despite the rich body of literature (see above), studies that aim to correlate the traits of a host-parasite relation, and, in this way, address the trade-off hypothesis, are scarce. Moreover no study that addressed this hypothesis has resorted to the model organism *D. melanogaster* despite its intensive use in immunity studies⁵⁵.

In this project, we aim to address this topic using a model system we have well established in the lab, composed of *D. melanogaster* and its parasite, the bacterium *Pseudomonas entomophila*.

In a first stage, we will expose an outbred population, by a natural route (ingestion), and measure virulence and transmission. We aim to establish a protocol that allows to measure virulence and transmission in the same population and generation.

In a second stage, we will use the optimized protocol and test different host genetic backgrounds. To this objective, we will infect the *D. melanogaster* Genetic Reference Panel (DGRP) lines with *P. entomophila* and measure the same traits as before. Taking advantage of the knowledge we have about the DGRP fly lines we will perform a subsequent genome-wide association study (GWAS) between the established phenotypes and underlying genetic variants (SNPs).

With this study, we aim to:

- 1) Produce a robust test to the trade-off hypothesis using the power of our *Drosophila-Pseudomonas* model, and
- 2) Establish the first association between host genetic architecture and transmission and virulence of its pathogen.

2. MATERIAL AND METHODS

2.1. The biological system

2.1.1. *Drosophila melanogaster*: Wild-type outbred population

An outbred population of *Drosophila melanogaster* was established in the laboratory in 2007, from 160 *Wolbachia*-infected fertilized females, collected in Azeitão, Portugal. Variability in this base population was assessed using multiple methods, based on 103 SNPs located in the left arm of the 3rd chromosome¹³. It contains high and relatively constant levels of polymorphism. The population was kept in laboratory cages for over 50 non-overlapping generations (generation time: three weeks) with high census (over 1500 individuals). Flies were maintained under constant temperature (25°C), humidity (60–70%) and light-darkness cycle (12:12), and fed with standard cornmeal-agar medium.

2.1.2. *Drosophila melanogaster*: white outbred population

An outbred white population of *Drosophila melanogaster* was established through introgression of the *white* gene into a wild-type outbred population during 12 generations using high census, in order to conserve the genetic variability of the population⁵⁶. The population was kept using the same procedure as described in 2.1.1.

Because we are using two different groups of individuals with two dissimilar purposes we need to be able to easily distinguish them. We tested different methods to distinguish them, like clipping wings⁵⁷, sterile pricking or painting the wings⁵⁷, but none was effective. Additionally, the first two approaches may involve the activation of the immune system, since there is damage to the body. Consequently, we used a receptor population with a distinct phenotype against which both control and infected groups can be tested. Therefore we used *white* outbred population. This population allows us to maintain the genetic variability of the donor outbred population and to distinguish the individuals for the two measurements.

2.1.3. *Drosophila melanogaster*: DGRP lines

The *Drosophila* Genetic Reference Panel (DGRP) lines were established from a natural population collected from Raleigh, North Carolina, USA, and inbred for 20 generations of full-sib mating, followed by random mating³⁹. Most lines have been completely sequenced^{39,43}. The DGRP is composed of more than two hundred isogenic lines, all of these lines have their genome sequenced and publically available so that Genome Wide Association Studies (GWAS) can be readily performed as to map phenotype to genotype.

The population of each DGRP line was kept in laboratory vials with small census (between 10 and 30 flies). The small census does not present a problem since these lines are inbred, hence almost do not have genetic variability. Consequently, there is no risk of inbreeding depression. Flies were maintained under constant temperature (18°C), humidity (60–70%) and light-darkness cycle (12:12), and fed with standard cornmeal-agar medium. The flies were flipped to new vials once every 21 days.

To select a subset of the DGRP, we used data from a study by Bou Sleiman (2015)³³ in which a collection of DGRP lines was orally infected with *P. entomophila* (Figure 1.3). From this data we selected lines that showed high, intermediate and low percentages of mortality upon infection. This way we expected to obtain information through the all spectrum of phenotypes.

2.1.4. *Pseudomonas entomophila*

P. entomophila was used to infect *D. melanogaster* populations. For each round of infection, bacterial pathogens were grown in Luria Broth medium (LB) inoculated with a single bacterial colony, taken from solid medium cultures grown from glycerol stocks kept at -80°C and streaked in fresh (1 week) Petri dishes supplemented with rifampicin (0.5 mg/ml). *P. entomophila* was prepared from an overnight

culture grown exponentially at 28°C, centrifuged and adjusted to OD₆₀₀=110. The *P. entomophila* strain existing in the lab was a generous gift from Bruno Lemaitre.

Moreover, the strain used is resistance to Rifampicin, which constitutes a major advantage to the project. Rifampicin is a potent and broad spectrum antibiotic used against bacteria. It inhibits the bacterial RNA polymerase by blocking the elongation during transcription⁵⁸. First, this resistance greatly reduces the risk of contamination because we can use the antibiotic to prevent it; second, it allows for the plating of the entire fly and we may have confidence that the only bacteria growing from a plated homogenate is the bacteria of interest.

2.2. Oral infection

2.2.1. Previous protocol to measure virulence

Adult mated females' flies with 4 to 6 days old were collected to vials containing clean food covered by a filter paper disc embedded in bacteria solution. The bacteria solution was prepared by mixing *P. entomophila* solution (OD₆₀₀=100) diluted 1:1 with sterile 5% sucrose solution¹³.

The control treatment, followed the same procedure except that the filter paper discs were soaked in a solution of sterile LB diluted 1:1 with sterile 5% sucrose and food colouring (Supplementary Figure 1).

Mortality of these flies was followed for 5 days and virulence measured as the number of flies that died after exposed to the pathogen.

2.2.2. New protocol to measure virulence

Previous protocols were developed to obtain a control mortality of ca.70%, 5 days post infection (dpi) in the control population. As one of the main objectives of our study, we wanted to decrease this control mortality. In order to increase the throughput of the procedure instead of using small vials (Supplementary figure 2A), we used bottles (Supplementary figure 2B). This alteration allowed us to increase the number of flies exposed to the pathogen by one order of magnitude. Moreover, we added a blue food dye (10 µl/ml) to the bacteria solution. This gives us a better control of ingestion at the individual and population levels.

Adult mated females' flies with 4 to 6 days old were collected 24 hours later (in order not to recover from any influence of the CO₂ used during collection), they were added to a bottle containing clean food for the flies to have ample access to food.

To orally infect the flies, bottles were prepared with cotton with 3ml of sterile water and covered for 24 hours by filter papers soaked with a *P. entomophila* solution (OD₆₀₀=110) diluted 1:1 with sterile 5% sucrose solution (adapted from¹³). Also, a food colouring in a ratio of 10 µl of dye for 1 ml of bacteria solution was added.

About 200 mated females were then added to the bottle containing the filter paper disc soaked with bacteria solution and transferred for 24 hours to a control environment of constant temperature (25°C), humidity (60–70%) and light-darkness cycle (12:12). After this time interval, the infected flies were separated to vials containing normal clean food (Protocol 4).

The control treatment, followed the same procedure except that the filter paper discs were soaked in a solution of sterile LB diluted 1:1 with sterile 5% sucrose and food colouring.

Mortality of these flies was followed for 5 days and virulence measured as the number of flies that died after exposed to the pathogen.

2.2.3. Protocol to measure virulence and transmission

2.2.3.1. *Drosophila melanogaster*: outbred population

Collection and infection was performed the same way as described in 2.2.2.

After 24 hours, 10 mated females flies of the outbred white population (receptor population) were transferred to each vials containing 10 infected flies of the outbred phenotype (donor population). The flies were transferred to the control environment for 24 hours. Afterwards, the flies were anaesthetized with CO₂ and separated by phenotypes (wild-type/outbred and white) to different 1,5 ml eppendorfs and kept on ice. Subsequently, the flies were sterilized following the protocol in Brummel⁵⁹ and each fly was individually homogenized in 100 µl of sterile LB. The homogenate was serially diluted and 4 or 5 µl droplets of homogenates were plated on LB agar plates, supplemented with 40 µg/ml Rifampicin and incubated at 30°C overnight. The number of viable cells within an individual was estimated by counting the number of colony-forming units (CFUs) after incubation. The dilutions to be counted were chosen so that the colony counts ranged between 10 and 100¹³.

This procedure allowed us to measure virulence, as the number of flies from the donor population that die after exposed to the pathogen for 5 days, and transmission, as the number of receptor flies that have bacteria after 24 hours in the same vial as donor flies.

2.2.3.2. *Drosophila melanogaster*: DGRP lines

The lines were prepared 30 days before infection. Adults from DGRP line stock were transferred to vials with fresh food and yeast and flipped to new ones every 24 hours for 5 days. To maximize the number of eggs laid, the flies were kept at 25°C. Afterwards, the adults were discarded. Once 10 days had elapsed, of the adults that eclosed, 30 females and 15 males were distributed over 3 bottles containing fresh food and yeast, on the ratio of 2 females per male. The bottles were transferred to 25°C for 4 days. After which the adults were discarded.

Collection and infection was performed the same way as described in 2.2.2. After 24 hours, 10 mated females flies of the outbred white population (receptor population) were transferred to each vials containing 10 infected flies of the outbred phenotype (donor population). The flies were transferred to the control environment for 24 hours. Afterwards, the flies were anaesthetized with CO₂ and separated by phenotypes (wild-type/outbred and white) to different 1,5 ml eppendorfs and kept on ice. Subsequently, the flies were sterilized following the protocol in Brummel⁵⁹. To quantify the number of receptor flies with bacteria, we adapted a commonly used bacterial growth protocol⁶⁰⁻⁶², and combined it with the protocol to plate flies¹³.

The new protocol uses 96-well microtiter plates and resorts to a smashes the flies. To quantify the number of receptor flies with bacteria we measured the Optical Density of each well. We measured immediately after smashing and at different time points. Any increase in the OD value is due to the presence of *P. entomophila*, owing to the resistance marker. As confirmation of this hypothesis, we plated, on agar plates supplemented with rifampicin, the solutions from wells in which the value was stable and others in which it increased. The results confirm that wells with stable values did not display any bacterial growth whereas wells with increase values exhibited CFUs (Supplementary Figure 11).

Consequently, flies were placed in a 2 ml 96-well plate with a metal orb and 50 µl of LB. The plate was then placed in the tressulizer and the flies were smashed automatically. Afterwards, instead of serially diluting and plating the homogenates, we transferred 10-15 µl of the solution to 40-35 µl of LB-rifampicin to each well in a microtiter 96-well plate. Subsequently we measured the Optical Density (OD) at 600 nm. Afterwards, we transferred the microtiter plates to a growth environment at 30°C.

Afterwards, at different time points, we measured the OD of each well on a microplate reader (Perkin Elmer Victor 3 Multilabel Plate Reader) (Supplementary Figure 10).

This procedure allowed us to measure virulence, as the number of flies from the DGRP population that die after exposed to the pathogen for 5 days, and transmission, as the number of receptor flies that have bacteria after 24 hours in the same vial as donor flies.

2.3. Genome Wide Association Study

The GWAS was performed after the measurement of the phenotypic traits in the DGRP lines. We used the phenotypic measures obtained from the virulence measurements and used the DGRPs database site to perform the GWAS^{39,43}.

2.4. Statistical analysis

All statistical analyses were performed using the software R v3.2.5 (2016)⁶³.

2.4.1. Survival curves and hazard ratios

The proportion of individuals surviving at day 5 after infection in each vial will be first estimate using the Kaplan-Meier method. Individuals alive at the end of the experiment, stuck in the food or escaped from vials during the period of observation were counted as censored observations¹³.

Due to differences in the positive controls in different tests with the DGRPs we used the Cox's proportional hazard model in order to compare the survival of each line with its own control, with the positive control of each test as baseline and replicate vial nested within line as a random variable¹³. The hazard ratio test calculates the probability of dying relative to the control^{64,65}. This test permits us to normalized and compared survival trajectories despite the differences in the positive controls^{13,14,66} (Supplementary figure 6-7).

These tests were done using the R libraries *lme4* (v0.999999, generalized and linear mixed models), *coxme* (v2.2, mixed effects Cox proportional hazards model) and *glht* (v1.2, multiple comparisons)¹³.

2.4.2. Genome Wide Association Study

The results of the statistical tests applied in the GWAS data was provided in the delivery of the results from the database site^{39,43}. No further analyses were performed in the dataset. For the GWAS a Manhattan plot of the *P*-values for the association between genomic variants in DGRP lines and phenotypic information was made using the R library *qqman*³³.

2.4.3. Graphic representation

All graphs were obtained using the software GraphPad Prism 6⁶⁷.

3. RESULTS

One of the main objectives of this project was to develop a protocol that allows measurement of virulence and transmission in the model organism *Drosophila melanogaster*. Thus we sought to adapt the established protocol in our laboratory^{13,47} with two main objectives in mind:

- i. measure both virulence and transmission in the same population and generation;
- ii. have 50% mortality in an outbred population, in order to obtain the best baseline for the ensuing study with the *Drosophila* Genetic Reference Panel (DRPG) lines;

3.1. Previous protocol

One of the most used protocols to study immunity in arthropods, focuses on the natural route of oral infection that pathogen takes to infect the host through the gut. This protocol, has been used in different studies, which provided a broad range of knowledge about *D. melanogaster* immunity^{13,33,47,54} (Protocol 1). Since this protocol is established in our lab¹³, we adapted it in order to develop a new protocol to measure virulence and transmission.

3.2. New protocol to measure virulence and transmission

3.2.1. Measurement of virulence: creating the 50% mortality baseline

In a first test, we performed the infection using both protocols, the established and our adapted (Figure 3.1). We were able to reproduce the control mortality levels described before using the old method at 66-70% mortality (dotted line). As for the control mortality in our new method, it reached about 30%, 5 dpi. Hence our alteration to the protocol decreases the mortality of the outbred control population as desired but to an excessive degree.

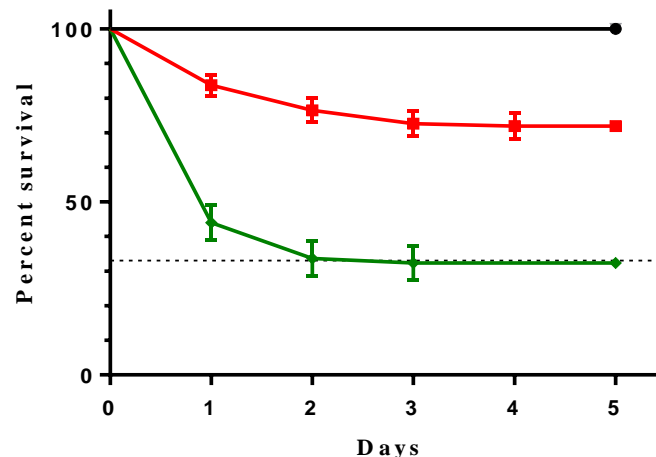


Figure 3.1. Survival Curve, Previous (P) vs Adapt (A) protocol. Trajectories of survival of outbred control population infected with entomopathogenic bacterium *Pseudomonas entomophila* using two different protocols, 5 dpi. For the P protocol: Gray line (behind black line), control treatment (n=100); Green line, infection treatment (OD₆₀₀=100) (5 replicates; n=100). For the A protocol: Black line, control treatment (n=159); Red line, infection treatment (OD₆₀₀=100) (n=153). Vertical bars correspond to standard error; the straight dotted line corresponds to the control percent survival of the previous protocol. For more information regarding statistical analysis see Supplementary table 1.

Subsequently, in order to increase the mortality as we wanted, we decided to change some steps of the protocol to try to raise mortality:

- i. separated the flies 24 hours before infection;

- ii. increased the concentration of bacteria solution to OD₆₀₀=110 instead of OD₆₀₀=100;

We tested these changes under different conditions inside the infection bottles. The simplest was adding cotton to the bottle with 3ml of sterile miliQ water, to keep the air moist for 24 hours. Other treatments consisted of i) a small portion of melted food, ii) the same melted food with dry yeast, iii) standard food and iv) standard food with dry yeast (Figure 3.2).

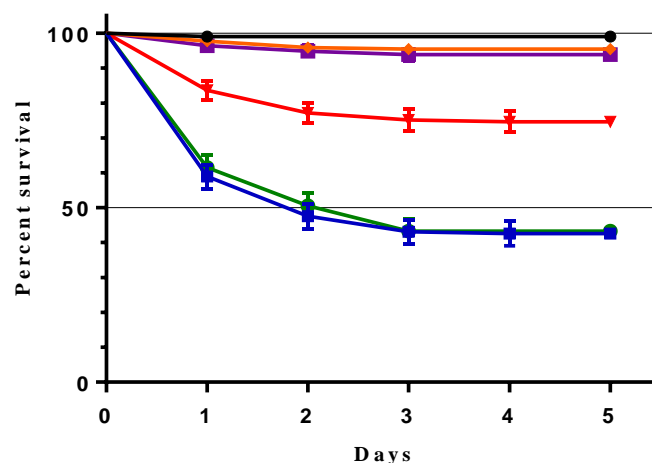


Figure 3.2. Survival curves under different feeding conditions. Trajectories of survival of outbred control population infected with *P. entomophila*, 5 dpi. Black line, control treatment (n=196); the other treatments were all infections: Blue line, cotton condition (n=202); Red line, melted food (n=201); Orange line, melted food plus yeast (n=219); Green line, standard food (n=194); Purple line, standard food plus yeast (n=194). Vertical bars correspond to standard error. For more information regarding statistical analysis see Supplementary table 2.

As can be seen in Figure 3.2, the conditions greatly influence mortality. In all cases, there is a significant increase in mortality compared to the control (Supplementary table 4). Additionally, according to these results, yeast appears to mitigate the effect of infection (Orange and Purple lines). The most important result is that the changes could increase the mortality to the desired baseline, around 50%. The conditions of cotton and standard food (Green and Blue lines) present a mortality close to the intended. Moreover, we found no significant difference between placing the filter paper disc on top of standard food or wet cotton ($p=0.8124$). These results suggest that there is no real influence of food on fly mortality besides the maintenance of moisture inside the bottle. Subsequently, we adopted the simplest protocol (Supplementary Figure 3).

3.2.2. The pathogen: dynamics and effect of *Pseudomonas entomophila* in infected flies

The main difference of our protocol to previous methods is the way by which the host is exposed to the pathogen. Importantly, we had to ensure the stability of the bacteria available in the medium over the course of the 24 hours (Protocol 2). Our results show, a significant decrease of quantity in the last hours (6 h vs 24 h, $p=0.0226$). However, we consider there is more than enough quantity of bacteria over the 24 hours of infection to expose all the individuals as their absolute numbers remain extremely high over the entire period (Figure 3.3).

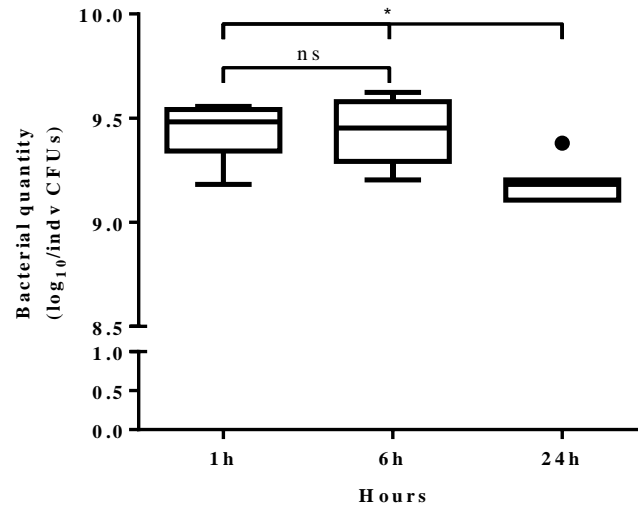


Figure 3.3. Quantity of bacteria in the filter paper discs through 24 hours. Bacterial quantities in individual filter paper discs (n=7 per time point) after dipping them in bacteria solution. The value of \log_{10} was calculated after counting the number of CFUs in a rifampicin agar plate, incubated at 30°C overnight. Boxplots include median, 25-75% percentile and whiskers represent 95% confidence interval. The black dot represents an outlier value. Note that the Y axis has a gap. ns p -value > 0.05; * p -value < 0.05. For more statistic information, see Supplementary table 3.

Next we measured bacteria inside infected flies. One new component of the new protocol was the addition of a blue food dye, to obtain a visible evidence that the flies ate the bacteria solution. To test if the presence of dye in the gut correlates with the presence of bacteria we separated flies according to the presence or absence of dye in the gut and afterward plated them (Figure 3.4).

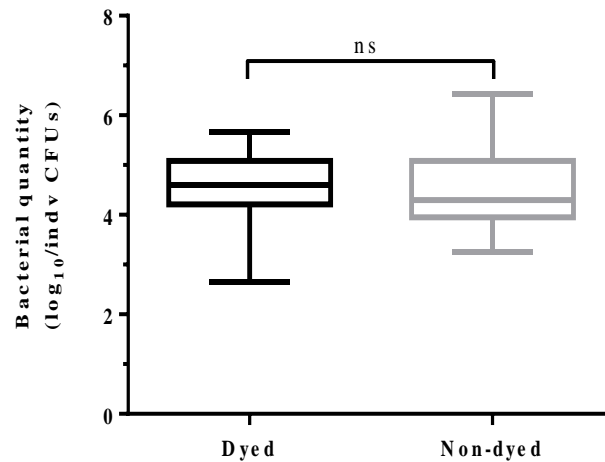


Figure 3.4. Food dye in the gut is a poor indicator of feeding. Bacterial loads in individual flies (n=20 per treatment) at 0h. Flies were separated by the presence (black box) or absence (grey box) of food dye in the gut. The value of \log_{10} was calculated after counting the number of CFUs on rifampicin agar plate, incubated at 30°C overnight. Boxplots include median, 25-75% percentile and whiskers represent 95% confidence interval. ns p -value > 0.05. For more statistic information, see Supplementary table 4.

We found no significant difference between dyed and non-dyed flies ($p=0.9028$) in the amount of bacteria they harbor. This shows that the blue food dye is not a good proxy for the infection status. Flies are able to clean *P. entomophila* from the gut after *ca.* 16 h³³. Moreover, food intake is blocked by *P. entomophila*^{33,49}. These two factors may explain the absence of dye in infected flies.

Given that the presence of blue dye is not a good indicator of infection status, we wondered what proportion of the 200 flies was infected. To determine this, we plated individually 50 flies per bottle, the maximum number of flies technically achievable. Moreover this test allowed us to assess the extent

of variation of bacterial load in infected flies (Figure 3.5). Although the maximum number of flies tested is only a portion of the infected population, the fact that all tested flies had bacteria gives us some confidence to consider that, if not all, almost all infected flies have bacteria.

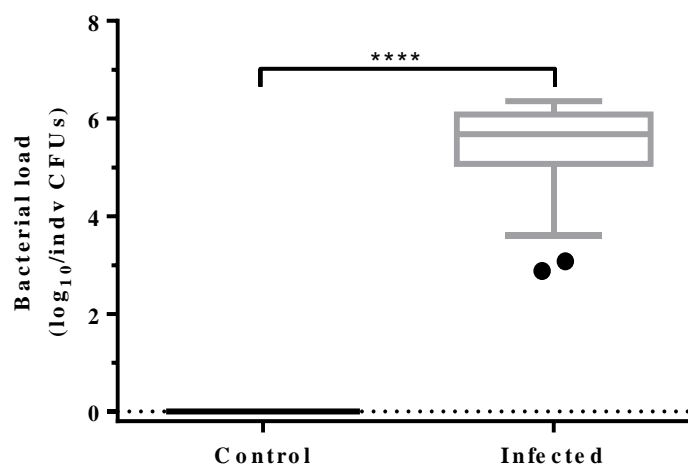


Figure 3.5. Bacteria load of infected flies. Bacterial loads in individual flies at 0 hours after infection. Control treatment (black box, n=10); Infection treatment (grey box, n=40). The value of \log_{10} was calculated after counting the number of CFUs on rifampicin agar plate, incubated at 30°C overnight. Boxplots include median, 25-75% percentile and whiskers represent 95% confidence interval. The black dot represents an outlier value. **** p -value < 0.0001. For more statistic information, see Supplementary table 5.

Above, we have determined the bacteria presence in the gut using a protocol (see M&M and Protocol 3), which removes bacteria associated to the surface of the flies. Our interest in bacteria that infect flies through the natural oral route has guided this choice. However bacteria on the outside the host may have some influence on transmission as they constitute a pool of available bacteria for cross-transmission⁶⁸. Therefore, to test for the presence of bacteria in the fly cuticula, we repeated the protocol but without sterilizing the flies.

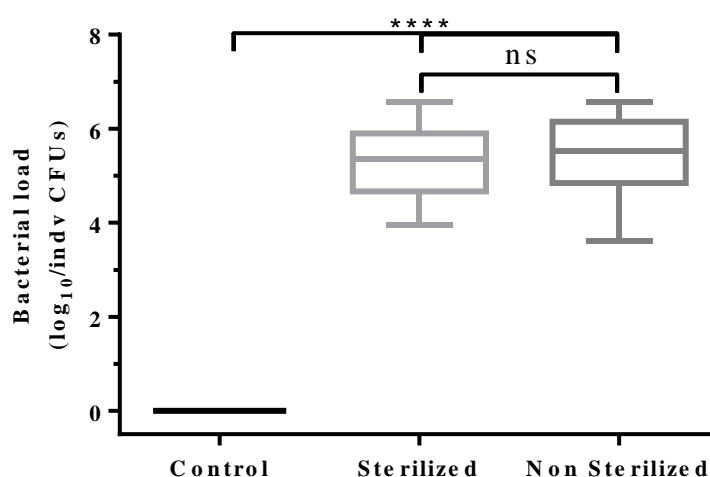


Figure 3.6. Amount of bacteria outside infected flies. Bacterial loads in individual flies at 0 hours after infection. Control treatment (n=10); Infection treatment: Clean, standard sterile flies (n=20); Non clean, non-sterile flies (n=20). The value of \log_{10} was calculated after counting the number of CFUs on rifampicin agar plate, incubated at 30°C overnight. Boxplots include median, 25-75% percentile and whiskers represent 95% confidence interval. ns p -value > 0.05; **** p -value < 0.0001. For more statistic information, see Supplementary table 6.

As before we found a significant difference between both treatments and the control (Figure 3.6; $p < 0.0001$). However, we could not detect significant difference between treatments ($p = 0.7853$). Even though there's a slight increase in the non-sterile flies (Mean: Clean: 5.35 and Non Clean: 5.42), the

quantity of bacteria that is transported on top of flies is not significant and should have no impact on transmission in the following steps of our experimental procedures.

3.2.3. Dynamics of *Pseudomonas entomophila* in infected flies

To understanding how the bacteria load changes through time, we plated infected flies in intervals of 24 hours for three days. Since almost all mortality occurs within the first 3 days, we should expect the relevant alterations in bacterial load to take place within this period.

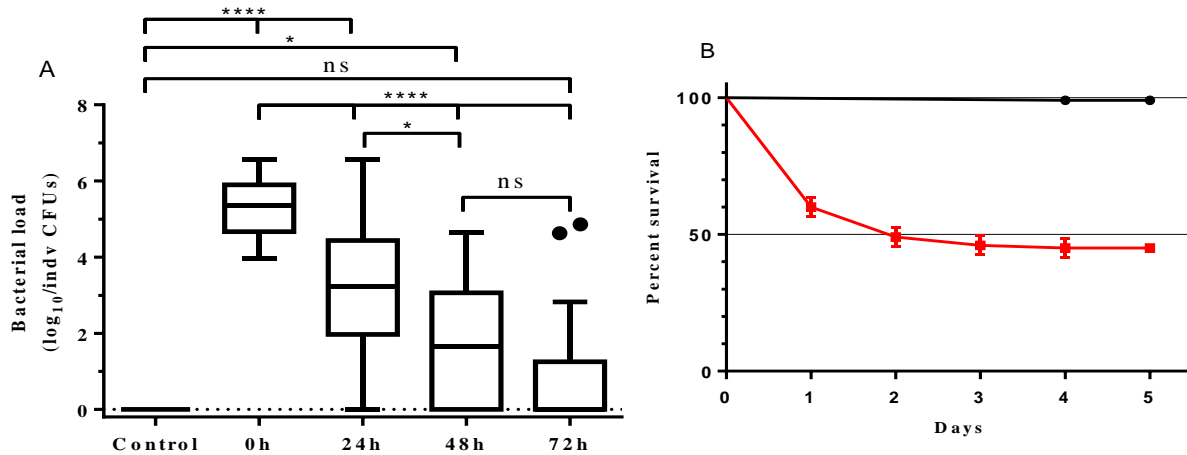


Figure 3.7. Time-course of the bacterial load inside flies over 72 hours and survival curve over 5 days. (A) Bacterial loads of individual live flies in the course of 72h after infection. Control treatment (n=10); for the infection treatment: 20 flies were plated individually at 4 time points. The value of log₁₀ was calculated after counting the number of CFUs on rifampicin agar plate, incubated at 30°C overnight. Boxplots include median, 25-75% percentile and whiskers represent 95% confidence interval. The black dots represent outlier values. (B) Trajectories of survival of outbred control population infected with *P. entomophila*, 5 dpi. Black line, control treatment (n=203); Red line, infection treatment (n=200). Vertical bars correspond to standard error. ns p-value >0.05; *p-value<0.05; ****p-value<0.0001. For more statistic information, see Supplementary table 7.

The first 24 hours corresponds to the period with the highest decrease in mean bacterial load (Mean (0 h)=5.356 log₁₀; (24 h)=3.018 log₁₀; 40% decrease). After 24 hours, some flies successfully eliminated the bacteria inside them (4 in 20). Despite the overall decrease in bacterial quantity, some individuals still present the highest amount of bacteria (more than 6 log₁₀) observed at time point 0 h. This is not the case at the 48 hours time point, when both the maximum quantity of bacteria per fly and the mean bacterial load decrease even further (Mean (48 h)=1.641 log₁₀). Moreover, the number of bacteria free flies increased (9 in 20). At the last time point both these trends continued with the mean bacterial load almost reaching 0 because 14 out of 20 flies have 0 CFUs and the bacterial load of the remaining flies is quite low (<10 on average). However, there are two outliers that present a bacterial load comparable with the prior time point (Figure 3.7.A).

Fly mortality takes place mostly in the first 24 hours after infection (Days 0-1, 40% decrease in live flies). After the first 24 hours the mortality rate decreased, up to 48 hours that represents just 11% decrease in survival, 3% until 72 hours, and 1% till the fifth day post-infection (Figure 3.7.B).

3.2.4. Transmission

3.2.4.1. Testing virulence in the receptor population

To measure transmission, we used a receptor population (*white*) that could be distinguished from the donor population

We then tested if the white mutation affected the phenotypes observed. We found a significant difference between the survival trajectories of the two populations ($p=0.0002$) when using our new adapt protocol

(Figure 3.8.A). Since this new protocol uses a food dye and the main difference between the populations are the eyes, we hypothesize that this might have some influence. For that reason we tested the populations using the previous protocol that does not use the food dye (Figure 3.11.B). The difference between the survival trajectories of the two populations was maintained ($p=0.003$).

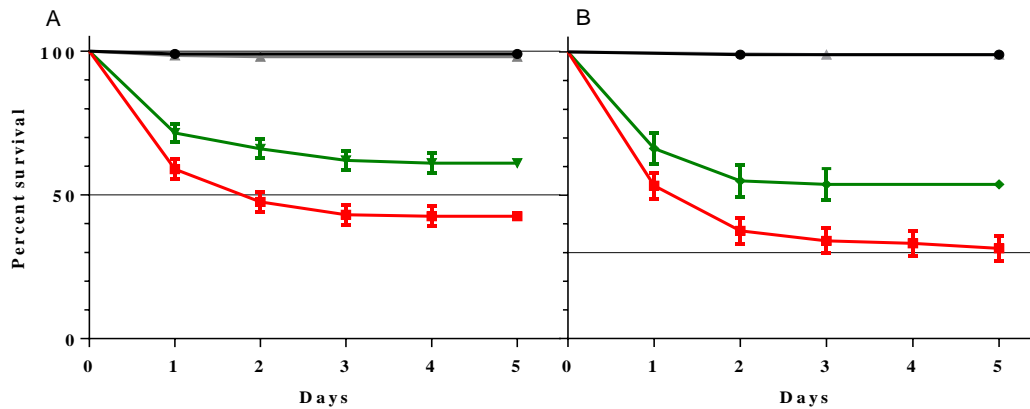


Figure 3.8. White outbred population survival upon *P. entomophila* oral infection. Trajectories of survival of outbred populations infected with *P. entomophila*. **(A)** Using the new protocol: Black line, wild-type outbred population, control treatment (n=196); Red line, wild-type outbred population, infection treatment (n=202). Grey line, white outbred population, control treatment (n=202); Green line, white outbred population, infection treatment (n=200). **(B)** Using the previous protocol: Black line, wild-type outbred population, control treatment (n=100); Red line, wild-type outbred population, infection treatment (n=120). Grey line, white outbred population, control treatment (n=100); Green line, white outbred population, infection treatment (n=80). Vertical bars correspond to standard error. For more information regarding statistical analysis see Supplementary table 8.

Regardless of the protocol we tested, there was a difference in the two populations regarding how they respond to infection. However, without other viable option, we used the white outbred population has the receptor population to measure transmission of the pathogen. Future studies to find other available options will be needed to optimize the protocol.

3.2.4.2. Measuring transmission

We tested transmission by mixing the infected *D. melanogaster* outbred control population with the uninfected white outbred population (Protocol 4). We determined survival and bacterial loads, as described before, in the receptor population 24 hours after joining the two populations (Figure 3.9).

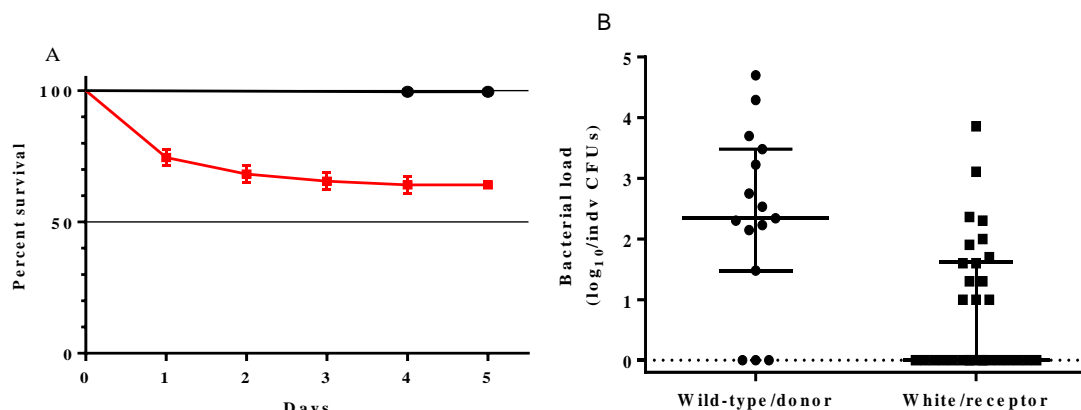


Figure 3.9. Horizontal transmission of *P. entomophila*. **(A)** Trajectory of survival of outbred population infected with *P. entomophila*, 5 dpi. Outbred control population: Black line, control treatment (n=201); Red line, infection treatment (n=201). Vertical bars correspond to standard error. **(B)** Bacterial load in individual flies at 24 hours post infection. Wild-type/donor population (n=15); White/receptor population (n=30). The value of log₁₀ was calculated after counting the number of CFUs on rifampicin agar plate, incubated at 30°C overnight. Scatterplots include median and whiskers represent 95% confidence interval. For more information regarding statistical analysis see Supplementary table 9.

Overall mortality in this test was relatively low. Regarding bacterial load in the receptor population (Figure 3.9.B), of the 30 flies from the receptor population tested for the presence of bacteria, 14 (47%) contained bacteria. In the donor population, of 14 tested flies, 3 were clean of bacteria after 24 hours and the mean and maximum values of bacterial load were around one order of magnitude below what we found in previous tests (see Figure 3.7.A). This result might be explained by some natural fluctuation of the infection protocol (Supplementary figure 1).

To correlate the two traits, we wanted to have measurements across the all spectrum of mortality. Because of the absence of available *P. entomophila* strains with different effects that we could use to measure various virulence percentages, we tested different quantities of bacteria to obtain different mortalities (Supplementary Figure 4).

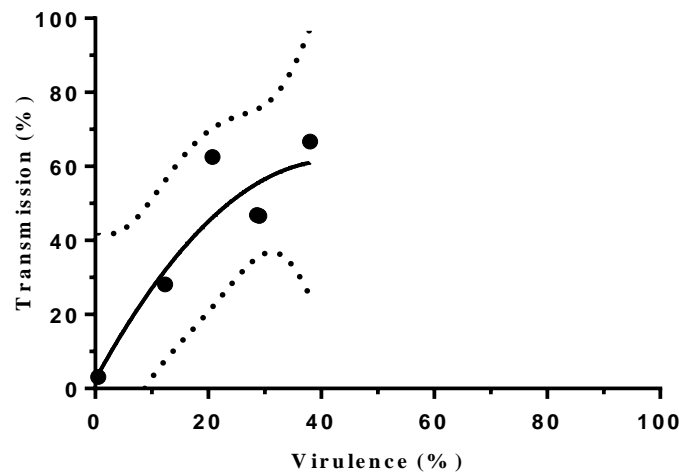


Figure 3.10. Trade-off hypothesis theoretical and experimental data. Plot of transmission and virulence percentage of the wild-type outbred population. Measurement of virulence of an infected population with *P. entomophila* and percentage of individual flies of the receptor population that tested positive for the presence of bacteria. For more information regarding statistical analysis see Supplementary table 10.

Due to lack of time we were unable to measure more than 6 points. Moreover, we were only able to measure transmission in cases with low mortality (Figure 3.10). As a result of this lack of data we can only do a comparison with the initial segment of the theoretical trade-off curve (Figure 1.1). Thus, to perform a stronger comparison between our system and the trade-off hypothesis, we require more data through the all spectrum of mortality.

3.3. The impact of host genetic background on virulence and transmission of *Pseudomonas entomophila*

The protocol we established was then applied to test how different host genetic backgrounds affected the measurement of virulence and transmission. To this aim, we infected several lines of the *Drosophila* Genetics Reference Panel (DGRP).

Previous studies tested the lines for the effect of harboring the natural endosymbiotic *Wolbachia* in survival to infection and they did not detect any influence in susceptibility^{33,69}. Likewise, the feeding behavior and the endogenous microbiota of the DGRP lines were evaluated for biasing the results and for none was found an impact in the observed differences³³.

We tested for differences in the initial pathogen load of infected DGRP flies. Overall, we did not see any differences in the initial pathogen load (Supplementary Figure 6). However, due to the small number

of lines and flies tested, in future studies a higher number of lines and flies should be tested to access if there are differences in the initial pathogen load.

3.3.1. Virulence

Using the measurements with our new protocol, we wanted to assess the differences in mortality between the two protocols. In each infection, additionally to the negative control we did a positive control that consisted in infecting outbred control population flies. This second control allows us to verify if the protocol was successful. In order to test the DGRP lines we divided them in small groups. These groups were composed of lines spread throughout the spectrum of mortality (Figure 3.11).

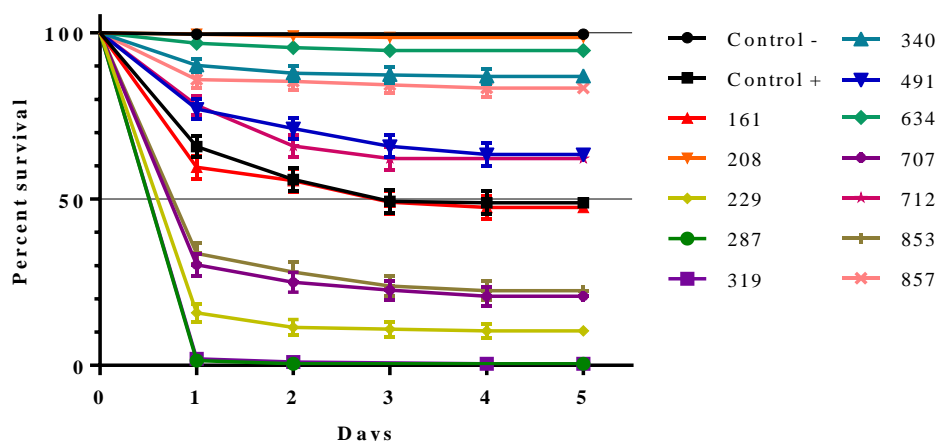


Figure 3.11. Survival curves of DGRPs lines upon oral infection. Trajectories of survival of populations infected with *P. entomophila*, 5 dpi. Black lines are the control treatments: circles for the negative control and square for the positive control. Other curves correspond to different infected DGRP lines. Vertical bars correspond to standard error. Supplementary Figure 6.

In some tests, the positive control did not have 50% mortality upon infection (Figure 3.11; Supplementary Figure 7). Hence we normalized the results for each infection with the positive control using the Cox's proportional hazard model.

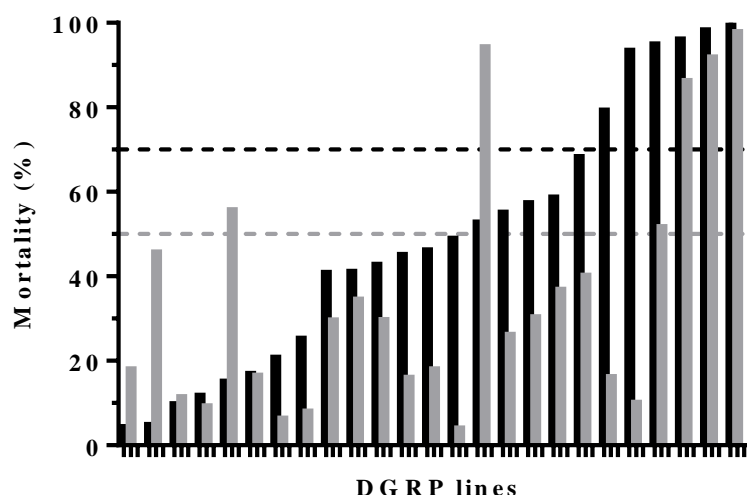


Figure 3.12. Contrasting survival analyses. The black bars correspond to the mortality percentage obtain by Bou Sleiman (2015)³³ and the grey bars correspond to the mortality generated in our project. The straight dotted lines corresponds to the control mortality rate of each protocol. For more information see Supplementary table 11.

For a better comparison between the data of our study and Bou Sleiman's, we only plotted results which the positive control follow the baseline of 50% mortality (Figure 3.12). Since the protocols have

different mortality control (our *ca.* 50%, Bou Sleiman *ca.* 70%), we were expecting differences in mortalities when comparing the DGRP lines. On the other hand we were expecting similar trend, whereas a line that is susceptible using a protocol should be susceptible using the other protocol as well. Indeed, in most cases we see this trend (Supplementary Figure 8A-8B), except in 4 lines where we have conflicting results (Supplementary Figure 8C).

3.3.2. Transmission

To avoid the technical limitations of the plating protocol, we develop a new method to assess the presence of bacteria in receptor flies. Our results confirm that wells with stable values do not display any bacterial whereas wells with increase values exhibited CFUs (Supplementary Figure 10-11).

We applied this new method to the DGRP lines. Again, because of lack of time we were unable to measure more than 7 points (Figure 3.13). We were able to measure transmission along most of the range of mortality. These initial data show an almost positive correlation between the two measurements (Figure 3.13; Supplementary Table 12). However due to the small number of data points we must take this result with caution. To draw a robust conclusion, we need to measure transmission in a greater number of DGRPs to reach a robust conclusion.

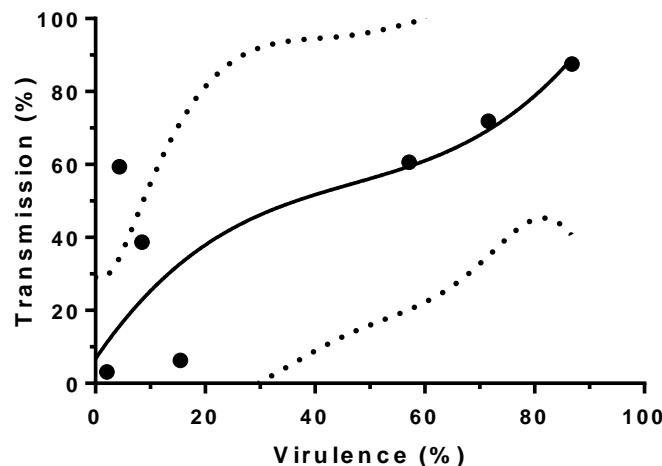


Figure 3.13. Measurements of virulence and transmission in DGRP lines. Plot of virulence and transmission percentage on subgroup of 7 DGRP lines. Measurement of virulence of an infected population with *P. entomophila* and percentage of individual flies of the receptor population that tested positive for the presence of bacteria. The black line represents the non-linear regression line and dotted lines indicate 95% confidence interval. For more information regarding statistical analysis see Supplementary table 12.

3.3.3. Genetic basis of susceptibility to infection

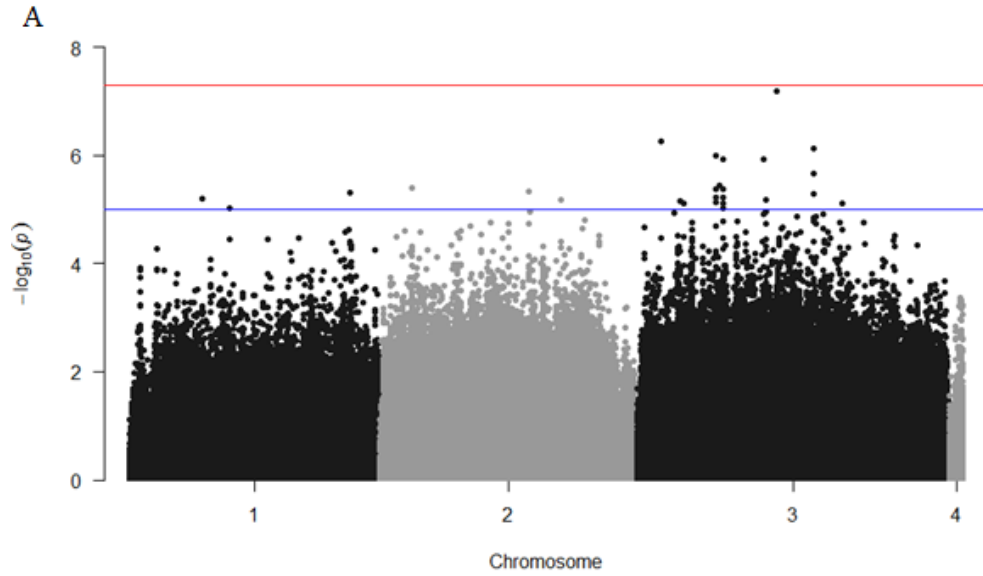
A previous study assessed mortality variation across DGRP lines and its underlying genetic basis upon oral bacterial challenge³³. Since our protocol is fairly different from previous methods, we wanted to study how our alterations influence the results and if there was any divergence with previous data.

To accomplish this objective, we performed a genome-wide association study (GWAS) on the mortality results. We obtained 26 quantitative trait loci (QTL) (Figure 3.14). Unexpectedly, there is no overlap in the QTL between our study and that of Bou Sleiman and colleagues³³.

The most significant QTL was located in an intron of the *so sondowah* (*sowah*) gene, which function is still unknown⁷⁰. As for the second most significant, it was located in an intron of the *zormin*, a gene expressed in the Z-disc and the M-line of muscles⁷⁰⁻⁷³. We also found the Heparan sulfate 6-O-sulfotransferase (*Hs6st*) gene, which has a function in FGF signaling during tracheal development in *D. melanogaster*⁷⁴ and a similar role has been found in mice⁷⁵. Moreover, in Zebrafish, this gene is related

to muscle development⁷⁶. CR31386 is the second gene with higher number of hits in our analysis (Figure 3.14B) and although its function is still unknown⁷⁰, it is downregulated in parthenogenetic females of *D. melanogaster*⁷⁷. As for the *shadow* gene, which shows the higher number of hits in our GWAS (Figure 3.14B), it is a member of the Halloween family coding for mitochondrial cytochrome P450 which mediates hydrolyzation reactions⁷⁸, and plays a role in determining the number of midline glial cells⁷⁹.

Due to the small number of data points we were not able to perform a GWAS in the transmission phenotype. Although this would be the most interesting and innovative result, since no previous study has study it before. In the future we aim to correct this.



B

Gene		Function
<i>sowah</i>	3L_12560926_SNP	Unknown ⁷⁰
<i>zormin</i>	3L_2137166_SNP	Expressed in the Z-disc and the M-line of muscles ⁷⁰⁻⁷³
<i>Hs6st</i>	3R_15764549_INS	FGF signaling during tracheal development; muscle development ⁷⁴⁻⁷⁶ .
	3R_15764622_INS	
	3R_15765156_SNP	
CR31386	3R_7106854_SNP	Unknown ⁷⁰
	3R_7106691_SNP	
	3R_7106859_SNP	
	3R_7106490_SNP	
<i>shadow</i>	3R_7703685_SNP	coding for mitochondrial cytochrome P450 ⁷⁸
	3R_7703761_SNP	
	3R_7702572_DEL	
	3R_7704565_SNP	
	3R_7703751_SNP	

Figure 3.14. GWAS results relative to susceptibility to infection. (A) Manhattan plot of the $-\log_{10} p$ -values (y axis) for the association between genomic variants in DGRP lines and *Pseudomonas entomophila* susceptibility. The x axis represents the genomic location (1-ChrX; 2-Chr2; 3-Chr3; 4-Chr4). Red line represents genome-wide Bonferroni significant threshold ($p = 6.6 \times 10^{-8}$) and blue line represents genome-wide Bonferroni significant threshold ($p = 5.6 \times 10^{-5}$). (B) Display of the genes more significant and with higher number of hits in the Manhattan plot.

4. DISCUSSION

Most organisms are involved in some form of symbiotic relation, whether as hosts or symbionts or both. Despite the difficulty that is to define a symbiotic relation, parasitism is one of the most common relationship between organisms⁴. Hence, the great focus in several studies. The main motivation to study parasitism is to further increase our understanding of the overall interaction. Additionally, in the long term, the potential to develop applications to medicine and agriculture, through new social policies and technologies¹⁹. This study focused on a constraint thought to be important during an infection: the trade-off between virulence and transmission^{6,18}. This model assumes that the evolution of higher rates of parasite reproduction and transmission must entail increase levels of virulence⁸⁰.

Our results show that virulence and transmission are positively correlated in both outbred and inbred lines in the pathosystem involving *D. melanogaster* and *P. entomophila*. This positive correlation is in strongly agreement with part of the trade-off hypothesis, which states that parasite virulence will not evolve to zero because of the benefits to the fitness of the pathogen⁶. Nonetheless, we do not see a saturation of the curve, also postulated by the trade-off hypothesis (Figure 1.1). Moreover, we analysed the bacteria during the time period of infection. We concluded that there is a correlation between the bacterial load in infected flies and the survival curve (Figure 3.7). Lastly, we studied the underling genetic bases of the virulence phenotype that we measured. None of the candidate genes we identified is associated with immunity or gut development (Figure 3.14). Overall, these results, although incomplete and vague, demonstrate the importance this new protocol may have to help shed light about the trade-off hypothesis.

4.1. Correlation between virulence and transmission - Outbred population

Due to the lack of available *P. entomophila* stains, we use the same method as in previous studies that produced different virulence levels through different strains^{22,23,28,29,81,82}, hence we resorted to different bacterial load to obtain different levels of harm. Despite successful, this method may have not been the more adequate, since, instead of varying the damage done to the host by the pathogen, and study how this correlates with transmission, we changed the bacteria quantity. Therefore, more or less transmission that we measure, may be solely due to have a higher or lower quantity of bacteria to be transmitted. At this point we cannot completely exclude this method, since the beginning of the trade-off curve is characterize by a positive correlation, which our data corroborates^{6,27-29}.

Analysing the measurements of virulence and transmission in an outbred population of *D. melanogaster* we detect a positive correlation between the two traits (Figure 3.10). This results are in line with previous studies that observe this positive correlation^{6,27-29}. The differences between the traits measured can be solely due to the different systems that are used in each study. Indeed, we expect to see a trade-off curve in different host-pathogen systems, since each system should have its own characteristics and several factors contribute to each trait^{18,24,83}.

Since we did not resort to any vector to transfer the pathogen to susceptible hosts, we may have expected differences between our results and previous studies. Despite previous tests showed that pathogens may influence the vector behaviour to maximize transmission^{29,84,85}, our method, by removing the vector factor, simplifies the system,. This way the relation between virulence and transmission that we measured in our model can be exclusively assign to our host and pathogen. Even with these differences we see the same positive correlation.

Moreover, we did not detect a saturation of the curve, like is postulated by the trade-off hypothesis and observed in the results of de Roode, Berenos and Doumayrou^{28,29,86}. Despite the differences between

systems stated before, this result may be because we did not recreate levels of virulence high enough that compromise transmission. Moreover, it could be due to the differences in the system and instead of a trade-off other correlation may characterize this specific system.

4.2. The dynamic of bacteria in infected flies

We found a declining trend in the bacterial load of infected flies over time (Figure 3.7.A). This decrease corroborates with a decrease in mortality through time. After 24 hours of infection, at time point 0 h, flies have the highest quantity of bacteria. After 24 hours, some flies were able to clean the bacteria from their gut. On the other hand, there are live flies with a high quantity of bacteria, similar to the quantity seen at 0 h. This suggests that in these flies the bacteria is able to multiply and maintain a high bacterial load. One possible explanation, is that these flies were not capable of containing the bacteria and consequently, avoid a systemic infection.

At the 48 h time point, two of the sampled flies have bacteria quantities compared to 0 h. We observe the same in the 72 h time point, represented by the two outlier values. In this 24 hours period, the percentage of individuals with high levels of bacteria, about 10% of the samples collected, was maintained. In spite of the evident decrease in the overall quantity in the population. This may constitute evidence of host tolerance to the pathogen^{87,88}, suggesting that the hosts were infected but were able to control or avoid the lethal effects instead of cleaning the bacteria.

Moreover, we observed that *P. entomophila* stays viable in dead hosts, which was previous unknown and may be a factor that influences, since it may explain linear relationship virulence-transmission that we observe in our results (Supplementary Figure 13). If the host death does not compromise transmission, then the correlation between virulence and transmission in our system may not be explained by the trade-off hypothesis. Although the results from the DGRP lines pointed to the possibility of an alternative model, Jensen and colleagues (2006)²⁷ observed a trade-off shape curve even though the parasite used needs to kill the host in order to be transmitted.

This data showed that the major decrease in survival is associated with the time period with higher quantity of bacteria in the population. Furthermore, the decrease in mortality can be correlated with the increase in number of flies without bacteria and the decrease in quantities in flies that still carry the parasite. However, around the 48 hour after infection we see a steady persistence of intermediate quantity of bacteria in a portion (10%) of the population. Has suggested before, this can be evidences of tolerance to a virulent parasite and flies are able to control or avoid the harmful interaction while still carrying the parasite. If this is proven right, it could be a strategy of the bacteria to transmit to other food sources and infect more susceptible individuals⁸⁹. Further studies are needed to better understand the dynamic of this bacterium in *D. melanogaster*.

4.3. How different host genetic backgrounds influence the trade-off?

The second main objective of this project was to explore how different host genetic backgrounds influence the phenotypes of virulence and transmission. We measured virulence in 92 DGRP lines. The mortality percentage ranged throughout the all spectrum (3% to 97%). Unfortunately, due to time constraints, we could measure the two traits only in 7 lines (Figure 3.13). Still, we measured transmission along most of the range of mortality (2.1%-86.8%).

Concerning virulence, our results recapitulated those observed by Bou Sleiman (2015), except for a few lines. These divergent results may be due to differences between protocols, for example in fly food or,

in the temperature to which the flies were maintained during the period of measuring virulence (our study: 25°, Bou Sleiman: 29°).

Since these lines can be considered a separation of a natural population genetical variability in individual lines, if a trade-off is observed in an outbred population, we may expect to broadly see this relation when using all the lines. Moreover, besides the trade-off curve, we may expect to see lines that do not fit in this hypothetical curve. The initial data show a positive correlation between virulence and transmission through all spectrum. Thus, we did not observe any saturation in the virulence curve. This result could, has said before, be due to the differences in our system, however, is more likely caused by the lack of sufficient points in the graph. Also, the model that defines the correlation between virulence and transmission in our system could not be a trade-off, which would have explained the lack of any saturation in the curve.

4.4. The genetic bases of virulence in *Drosophila melanogaster*

The last important objective of this study was to use the data of virulence and transmission to perform a GWAS. This would be done on each subset of measurements and to establish a correlation between the traits. Because of the reduced number of DGRP lines in which we were able to measure transmission we could not perform a GWAS on these data. Consequently, we were also unable enquire about a correlation between the traits. Unfortunately, we were only able to study the genetic of virulence.

The results obtained in the GWAS were unexpected^{33,48,89}, since the significant hits were almost exclusively in genes without known function. In addition, those for which the function is known do not appear to have any relation with immunity or with the gut. The only positive result with some kind of correlation, is the *Hs6st* which is related with tracheal development. This lack of candidate genes was already seen in the study of Bou Sleiman (2015) and in our case, can be further explained by the reduced number of lines tested (92 in 205). Moreover, the divergence between our results and those obtained by Bou Sleiman (2015), may be explained by the differences in the protocols. Furthermore, the use of hazard ratio values instead of mortality percentage may had influenced the results from the GWAS. Since the hazard ratios variation is quite small (-2.82 to 1.67) when compared to the mortality (3% to 97%).

4.5. Conclusion and future perspective

Despite the lack of results and setbacks, we can take some positive conclusions:

We were able to measure virulence and transmission using *D. melanogaster*. Moreover, we develop a method to orally infect an outbred population of *D. melanogaster* with a baseline of 50% mortality. This offers an excellent baseline for future studies that aim to answer questions related with oral infection using this system.

About the bacteria, we now know that we can use the standard fly food has a vector to infect *D. melanogaster*. This can be achieved even with a bacterium that is not adapted to this medium. Moreover, the possibility that bacteria in dead flies can be transmitted may influence the relation between virulence and transmission.

We also found that virulence of a parasite in a genetically variable population is the result of an admixture of different susceptibilities to infection. Therefore, the host genotype should be taken into consideration in future studies of this topic. The use of the DGRPs may help us to better comprehend how virulence and transmission are correlated in this model system and what genetic bases underling

this correlation. Moreover, possible outlier values, that do not fit in the model of an outbred population, if found, can give new knowledge about this relation.

In the future, we suggest the development of *P. entomophila* variants with different levels of virulence. This could be obtained by increasing mutation rate through chemical or stressful conditions^{48,90}. Subsequently, use the same bacteria quantity of these strain and study how this affects transmission in our outbred host population.

In future projects, we suggest that a higher number of DGRP lines should be tested. Further studies using all lines will allow to completely decompose the importance of the host genetics to the variance of specific traits during infection. Moreover, performing GWAS on the measured phenotypes, will allow the identification of important genes that contribute to this variation. Furthermore, we propose that instead of hazard ratios, the mortality percentages should be used to perform the GWAS.

Additionally, to test tolerance in infected flies, we propose that future studies measure the bacterial load dynamic past the 72 hours. If the bacteria persist in the population for a long period, even after the flies are transferred to clean food, it would support the tolerance hypothesis. On the other hand, if this is not observed, the high quantity observed in these results (Figure 3.7) may be due to some delay in cleaning. If we can prove that *D. melanogaster* is able to tolerate *P. entomophila* this may influence transmission⁸⁷.

Furthermore, we propose that future projects test the importance of the parasite to the variance of virulence and transmission^{31,36,91}. This could be assessed by infecting an inbred line, ideally with a control mortality close to 50%, with parasite strains with different levels of virulence, like the mutants developed by Peter Liehl (2006)⁹² and Isabelle Vallet-Gely (2010)⁹³. This way, hopefully several levels of mortality would be measured in the same host background. Hopefully, this will allow to measure just how much variance in virulence and transmission is due to the parasite. Finally, by identifying the genetic differences in these strains, one can expectantly identify unknown genes, first in *P. entomophila* and lately in others pathogens, related with variation on virulence levels. This way in addition to solving some problems we faced, there would be a stronger parallel between this study and the one to which Anderson and May based the trade-off hypotheses^{6,18,20,21}.

Finally, the positive correlation between virulence and transmission is often assumed to derive from a positive relationship of each of these traits with within-host replication: parasites with high within-host replication rates are often assumed to cause higher virulence and experience higher transmission^{6,94}. We proposed that future studies analyse if the same correlation is observed in our system.

In conclusion, this project shows that even in an established and largely used pathosystem there is still much to discover. The protocol that we now establish, although still needs more data, optimization and further tests, fulfils the need for a protocol to measure virulence and transmission in a horizontal transmission non-vector parasite. Our expectation is that this new protocol leads to more empiric studies and to a better understand of this complex relation that is the transmission-virulence trade-off hypotheses and more fundamentally the parasitic interaction.

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ANNEX

Protocols

Protocol 1. Protocol for oral infection with *Pseudomonas entomophila*

- 1) Select a protease positive clone (clear colony) and inoculate it in 40 ml of LB in a at least 200 ml Erlenmeyer and make an overnight culture at 28°C;
- 2) Make an overnight culture (16 to 24 h) by diluting the pre-culture 1/16 in a final volume of 400 ml in a at least 2 L bottle;
- 3) Pellet the cells by centrifuging *ca.* 2500g, 15', 4°C;
- 4) Remove almost all the supernatant and resuspend the pellet in the remaining medium;
- 5) Adjust the pellet to the required OD:
 - a. 100 OD for *ca.* 66% mortality
 - b. 50 OD for <50% mortality

Note: don't remove all the supernatant to avoid self lysis of the cells. Pe pellets sometimes undergo self-lysis anyway.

- 6) Sort the flies into fresh vials / bottles;
- 7) Prepare a 50:50 bacteria suspension / sucrose solution;
- 8) Prepare the filter paper so that it will completely cover the food surface;
 - a. Cut the paper to the required circle size;
- 9) Place the filter paper in new food vials, completely covering the food;
- 10) Dispense the bacteria suspension on top of the filter paper;
 - b. 150 µl;
 - c. Do not use excess suspension, as the flies will stick to the liquid;
 - d. Adjust the filter paper, so that the flies are not able to access clean food;
 - e. Always mix the bacteria suspension, as it tends to deposit;
- 11) Flip the flies from the uncontaminated food vials into the bacteria covered food;
- 12) After 24 h flip back the flies into to the uncontaminated food vials.;
- 13) Follow mortality for at least 5 days.

Protocol 2. Protocol for counting bacteria in a filter paper disc

- 1) Prepare a bacteria solution with: *P. entomophila* OD100-110 and dilute 1:1 in 5% sucrose. Add 10 uL/ mL solution of food dryer.
- 2) In one bottle, place cotton with 3 mL of water on the botton.
- 3) Make discs of filter paper (4,8-5 cm diameter) and dip them in the bacteria solution, both sides, and place the disc in the bottle.
- 4) 1, 6 and 24 hours after dipping collect the discs, place them in 20 mL of LB and shake the vial for 5 min.
- 5) Serially dilute the LB with bacteria (1:10; 1:100; 1:1000) in ependorfs by adding 100 µL of the solution containing bacteria to 900 µL of LB.
- 6) Place 5 uL of each dilution in petri dishes with LB+rifampicin.
- 7) Incubate plates at 30°C overnight.
- 8) Count the number of CFUs.

Protocol 3. Protocol for plating flies

Protocol adapted from the paper “*Drosophila* lifespan enhancement by exogenous bacteria”⁵⁹.

- 1) Flies are infecting following one of the protocols described in ‘**Protocol 1**’
- 2) After the infection transfer 10 flies to a eppendorf and place it in ice;
- 3) Wash the flies in 50% bleach for 2 min with agitation;
- 4) Then washed twice in 70% ethanol and twice with sterile, distilled water;
- 5) Then the flies are placed in a filter paper for absorption of remaining liquids;
- 6) Transfer a single fly or a pool of flies (3, 5 or 10) to clean eppendorfs;
- 7) Add 100 μ L of LB;
- 8) Smash the flies;
- 9) Serially dilute the LB with bacteria (1:10; 1:100; 1:1000) in eppendorfs by adding 10 μ L of the solution containing bacteria to 90 μ L of LB;
- 10) Place 5 μ L of each dilution in petri dishes with agar+rifampicin;
- 11) Incubate plates at 30°C, overnight;
- 12) Count the number of CFUs.

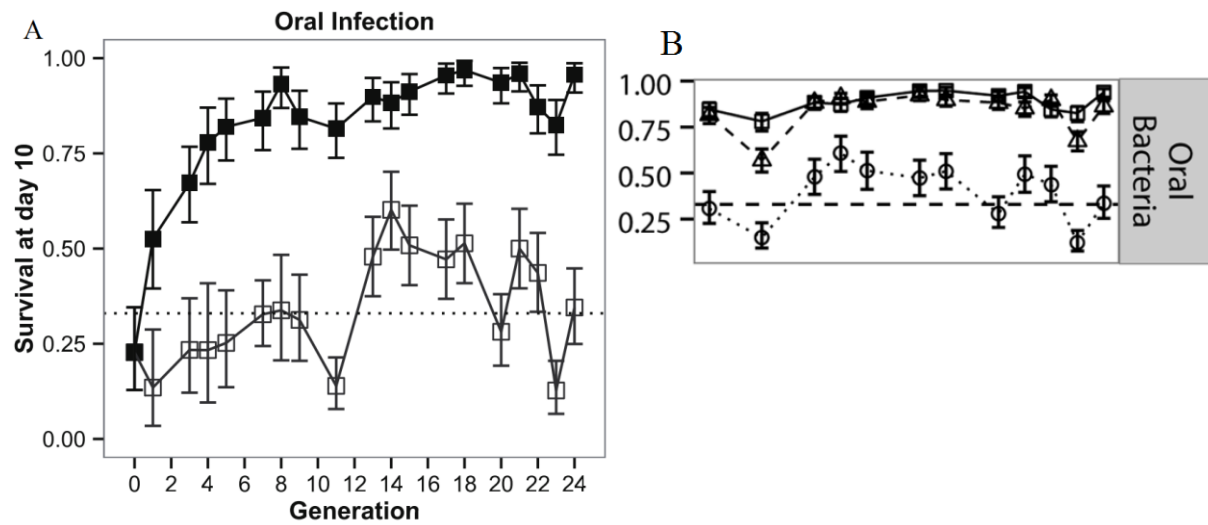
Protocol 4. Protocol to measure virulence and transmission – plating

- 1) Prepare the bacteria solution for oral infection;
- 2) Separate the females from the males and put them in a bottle containing clean food.
 - a. Discard the males; the adults must have between 4 and 6 days old.
 - b. Use only mated females.
- 3) Put a piece of cotton inside another empty bottle (the cotton must cover most of the bottom) and add 3 mL of sterile water;
- 4) Prepare a filter disc paper that completely covers the bottle ground;
- 5) Put the bacteria solution in an small empty Petric plate;
- 6) Dip the filter paper disc in the bacteria solution, both sides;
- 7) Then transfer the filter paper disc to the bottle containing the wet cotton and cover the bottom; clean the bottle walls with a piece of paper;
- 8) Transfer the flies to the bottle containing the filter paper disc and the cotton;
- 9) Wait 24 hours;
- 10) Anesthesia the flies with CO₂ and transfer them to a scope;
- 11) Separate the flies to vials, 10 flies in each one;
- 12) Add 10 receptor flies (White population) to the vials containing donor flies (Outbred population);
 - a. Add only to half the replicates;
- 13) Follow mortality for 5 days;
- 14) 24 hours after transferring the flies, plate recptor flies from three different replicates following the protocol for plating flies (**Protocol 3**);
 - a. the replicates must be composed of the extremes and an intermediate level or mortality observed;
- 15) After the overnight grow of the bacteria in the plates, count the CFUs;
- 16) The presence or absence of CFUs will allow to measure transmission and pathogen load.

Protocol 5. Protocol to measure virulence and transmission – OD measurement

- 1) Prepare the bacteria solution for oral infection (**Protocol 1** (steps: 1-5));
- 2) Separate the females from the males and put them in a bottle containing clean food.
 - a. Discard the males; the adults must have between 4 and 6 days old.
 - b. Use only mated females.
- 3) Put a piece of cotton inside another empty bottle (the cotton must cover most of the bottom) and add 3 mL of sterile water;
- 4) Prepare a filter disc paper that completely covers the bottle ground;
- 5) Put the bacteria solution in a small empty Petric plate;
- 6) Dip the filter paper disc in the bacteria solution, both sides;
- 7) Then transfer the filter paper disc to the bottle containing the wet cotton and cover the bottom; clean the bottle walls with a piece of paper;
- 8) Transfer the flies to the bottle containing the filter paper disc and the cotton;
- 9) Wait 24 hours;
- 10) Anesthetize the flies with CO₂ and transfer them to a magnifier;
- 11) Separate the flies to vials, 10 flies in each one;
- 12) Add 10 receptor flies (White population) to the vials containing donor flies (Outbred population);
 - a. Add only to half the replicates;
- 13) Follow mortality for 5 days;
- 14) 24 hours after transferring the flies, plate receptor flies from three different replicates;
 - a. the replicates must be composed of the extremes and an intermediate level of mortality observed;
- 15) Sterilize the flies (**Protocol 3** (steps 3-5));
- 16) Spoon flies to a 2 ml 96-well plate;
 - a. Use a tweezer to place a fly in each well;
- 17) Add a sterilized metal orb to each well;
- 18) Add 50 µl of sterilized LB to each well;
- 19) Seal the plate;
- 20) Smash the flies:
 - a. Tissueizer: 20 rps, for 2.30 min;
 - b. Centrifuge the plates: 1800 rpm for 1 min;
 - c. Repeat this three times;
- 21) Then transfer 5-10-15 µL of the homogenate to a 96-well microplate;
 - a. Microplate contains sterilized LB for serially dilutions followed by plating;
 - b. Microplate contains sterilized LB+rifampicin to measure Optical Density;
- 22) Measure the OD using the Victor 3 Machine;
 - a. Measure the OD₆₀₀ of each well three times.
- 23) Transfer the microplate to an incubator at 30°C;
- 24) After 60 hours measure OD using the same machine and protocol.

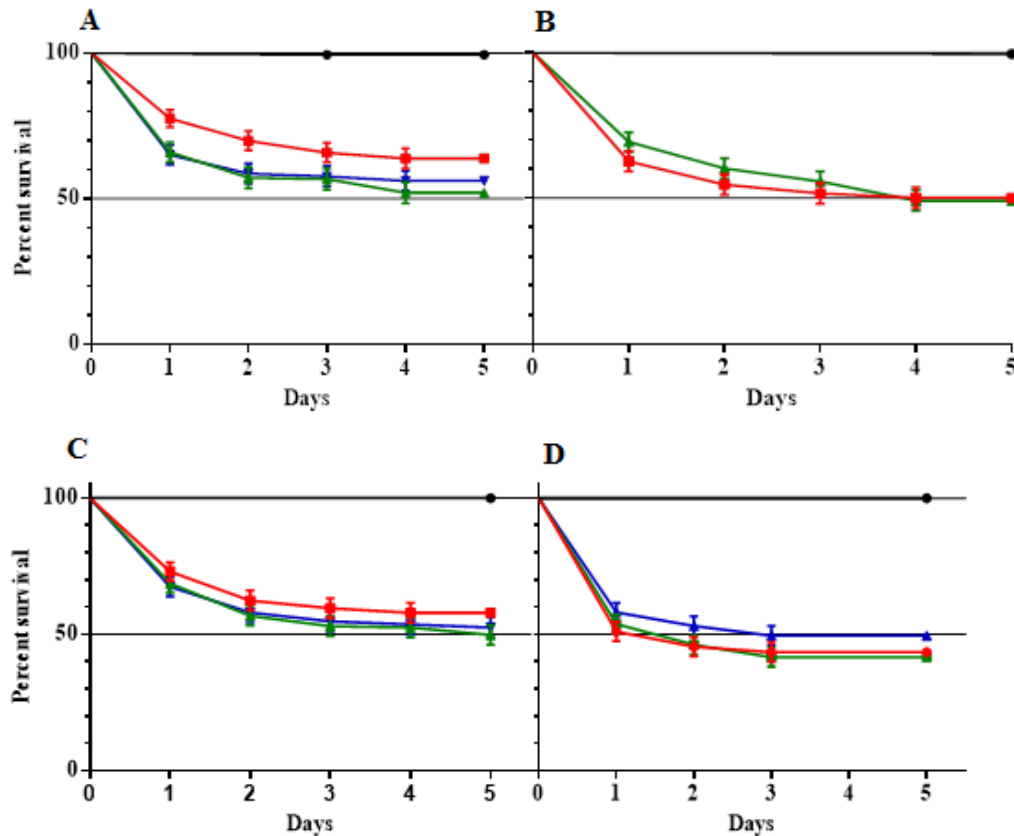
Supplementary Figures



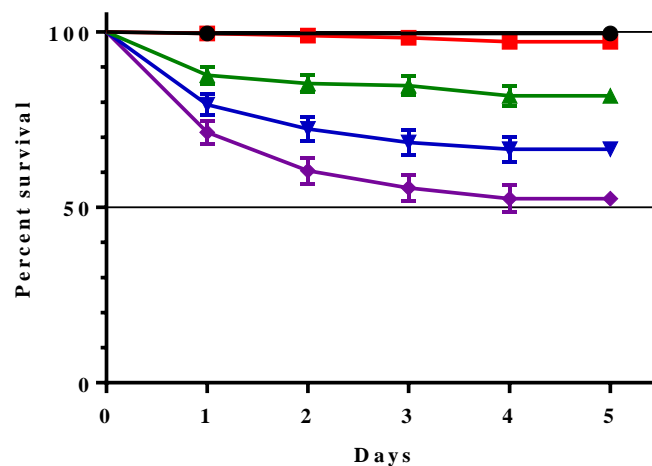
Supplementary Figure 1. Taken from Martins (2013)² (A) and Faria (2015)⁶⁸ (B). Experimental evolution trajectories of outbred *D. melanogaster* populations with *P. entomophila* oral infection. (A) Immediately after the experimental evolution. (B) In relaxed-selection maintenance. Black lines correspond to evolved lines and white lines to control populations. The vertical bars correspond to standard error across means of replicate lines.



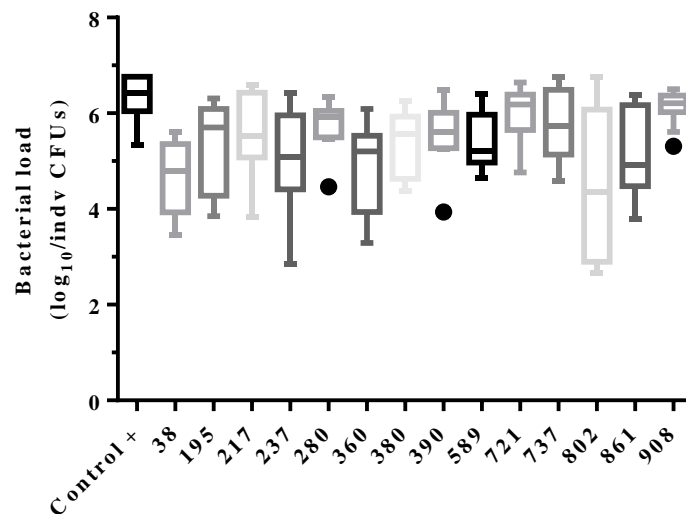
Supplementary Figure 2. Images of (A) vials and (B) bottles used for maintenance and handling of *D. melanogaster*



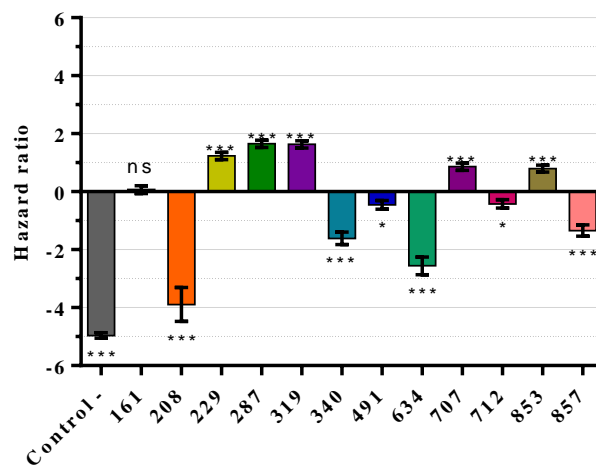
Supplementary Figure 3. Survival curve, variability of the new protocol. Trajectories of survival of outbred control population infected with *P. entomophila*, 5 dpi. Panels A and B correspond to two experimental replicates. **(A)** Black line, control treatment (n=193); the other curves correspond to different replicates of infection: Red line (n=196); Green line (n=194); Blue line (n=203). **(B)** Black line, control treatment (n=201); the other curves correspond to different replicates of infection: Red line (n=201); Green line (n=199). **(C)** Black line, control treatment (n=193); the other curves correspond to different replicates of infection: Red line (n=178); Green line (n=185); Blue line (n=181). **(D)** Black line, control treatment (n=193); the other curves correspond to different replicates of infection: Red line (n=198); Green line (n=197); Blue line (n=198). Vertical bars correspond to standard error. For more information regarding statistical analysis see Supplementary table 13.



Supplementary Figure 4. Survival curve, using different bacteria quantities. Trajectories of survival of outbred control population infected with *P. entomophila*, 5 dpi. Black line, control treatment (n=205); the other curves correspond to infections with different bacteria loads: Red line, OD₆₀₀25 (n=202); Green line, OD₆₀₀50 (n=202); Blue line, OD₆₀₀75 (n=188); Purple line, OD₆₀₀110 (n=199). Vertical bars correspond to standard error. For more information regarding statistical analysis see Supplementary table 14.

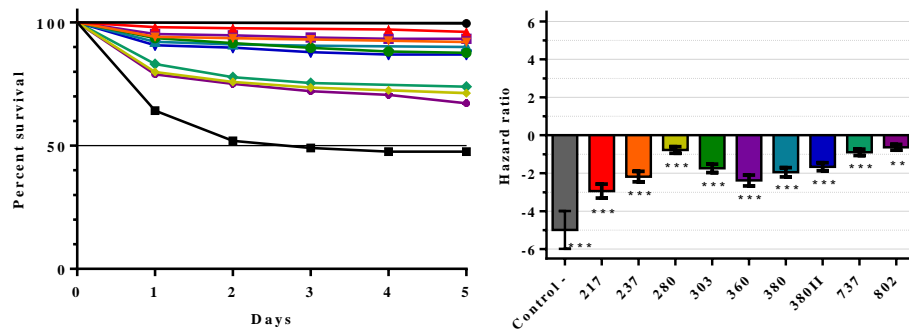


Supplementary Figure 5. Initial pathogen load of infected flies

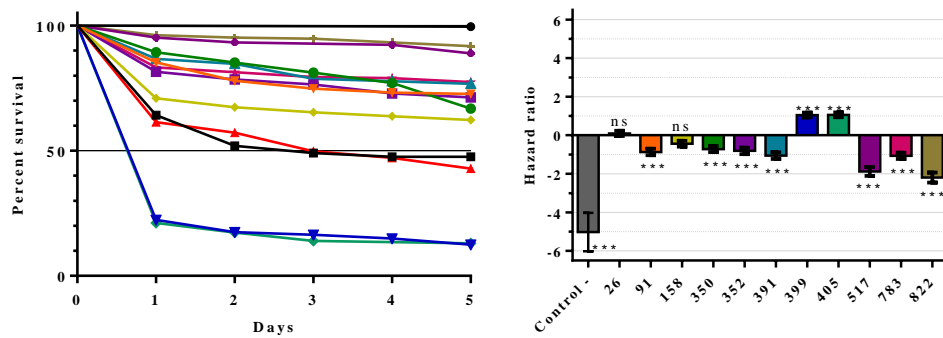


Supplementary Figure 6. DGRP lines susceptibility to oral infection with *P. entomophila* from Figure 3.11. Hazard ratios between survival percentages of flies of different DGRP lines. Positive control mortality curve functions as baseline. Vertical bars correspond to the standard error of the mean of the estimated hazard ratios. ns $p > 0.05$; * $p < 0.05$; *** $p < 0.001$.

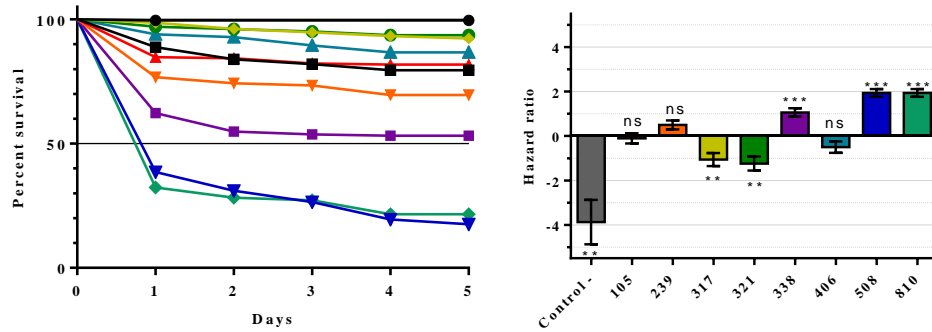
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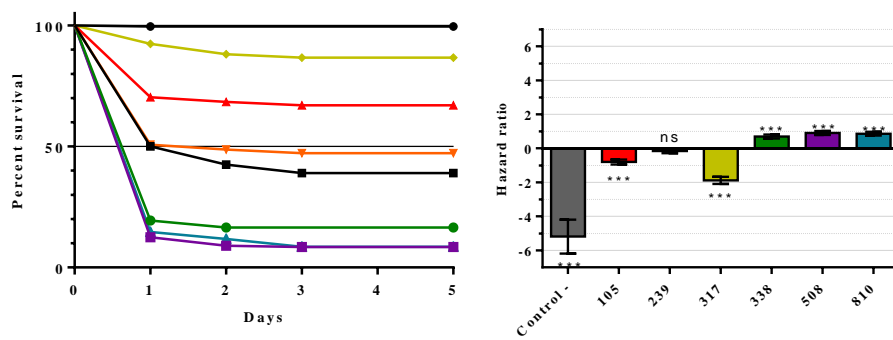
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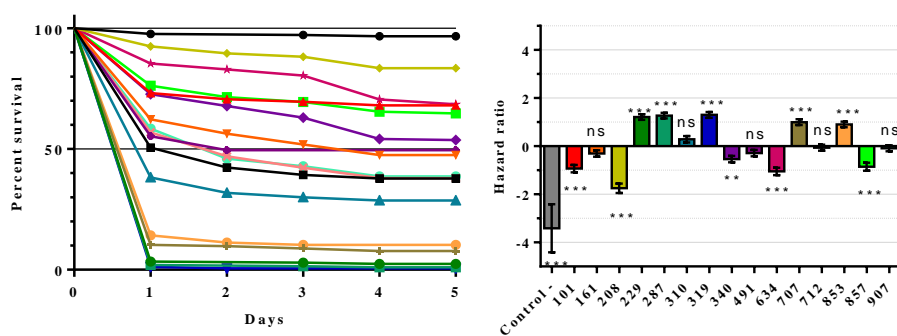
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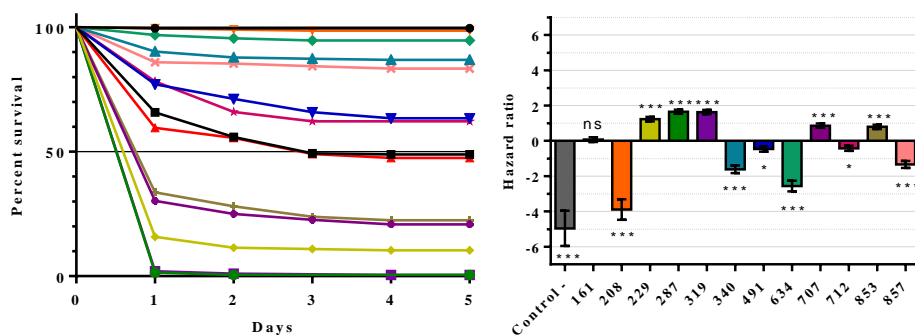
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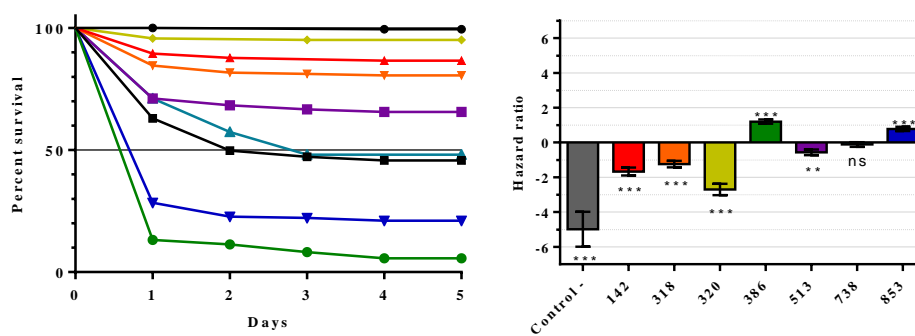
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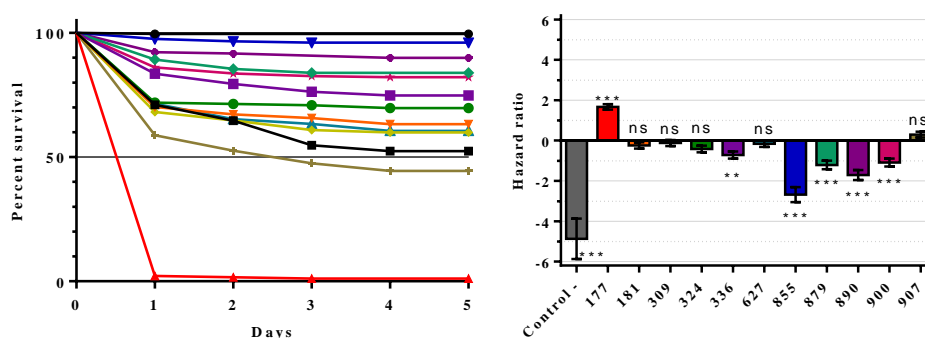
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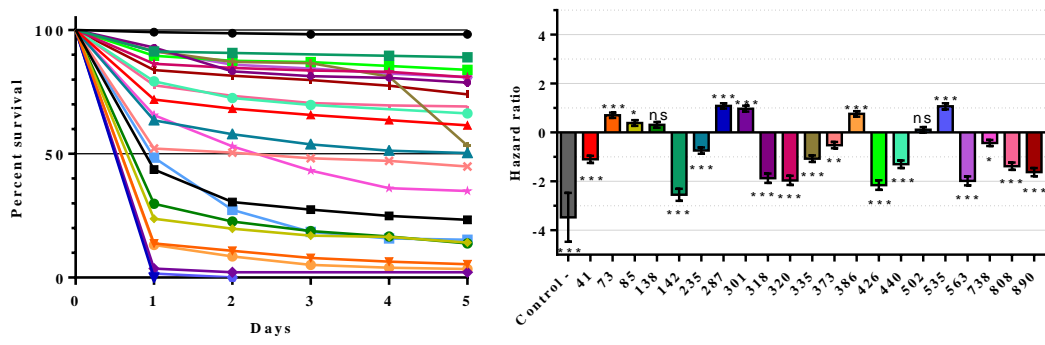
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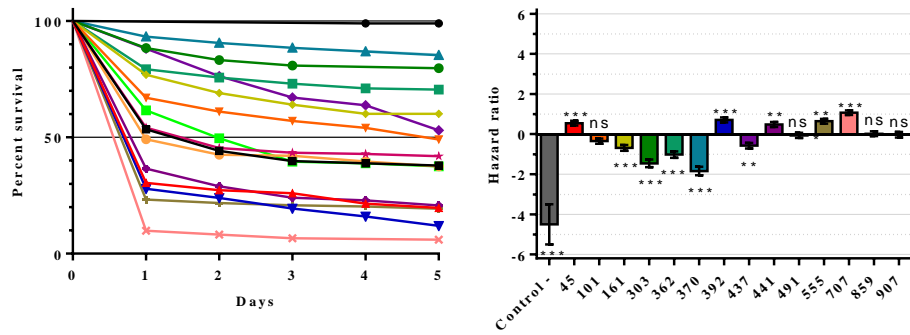
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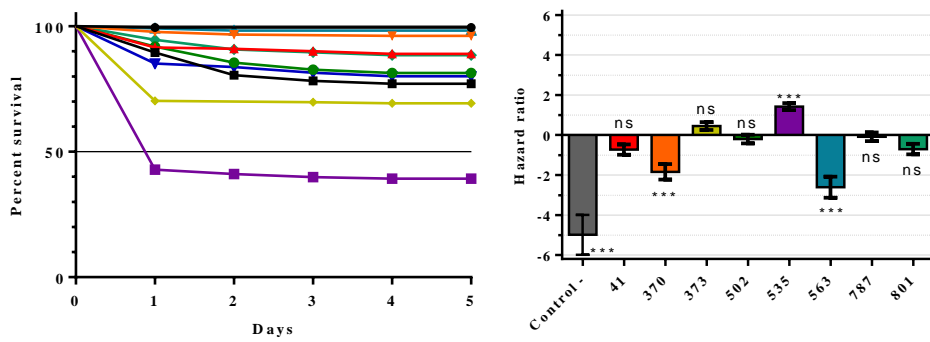
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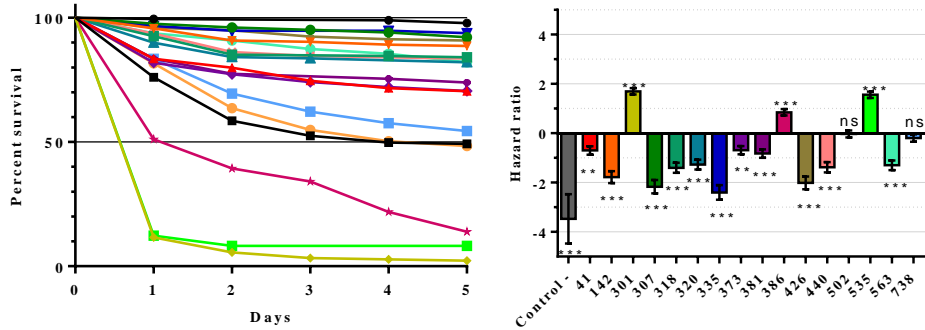
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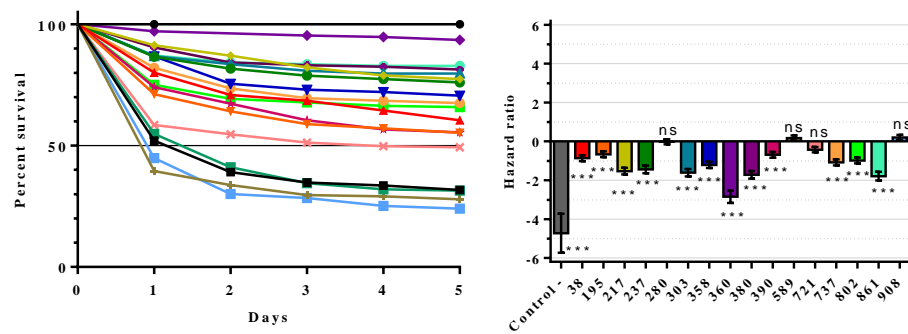
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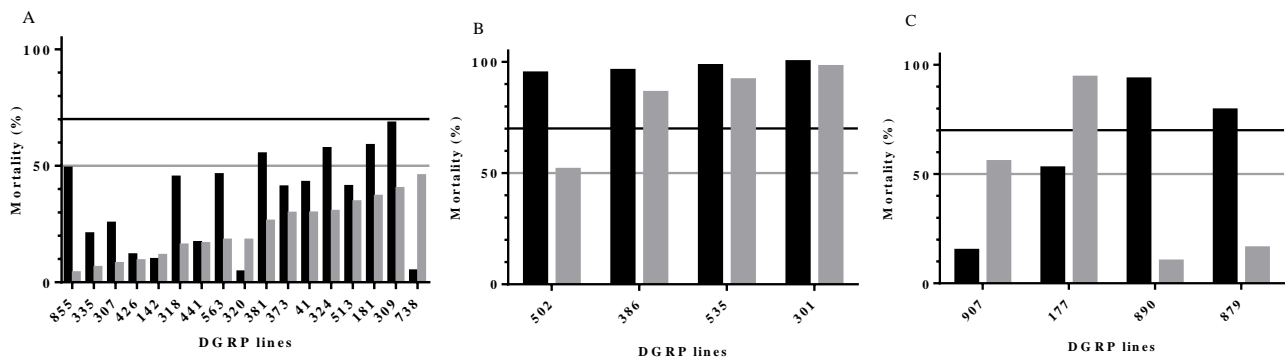
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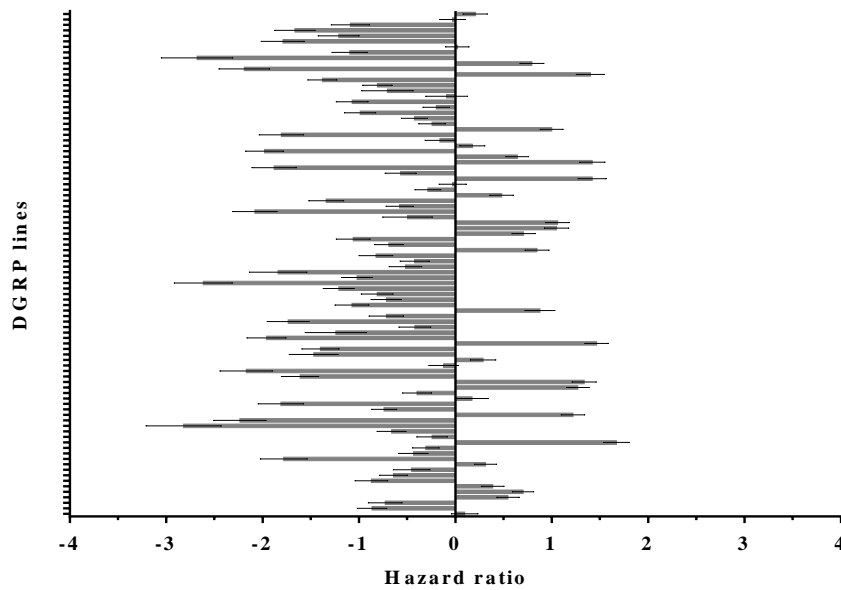
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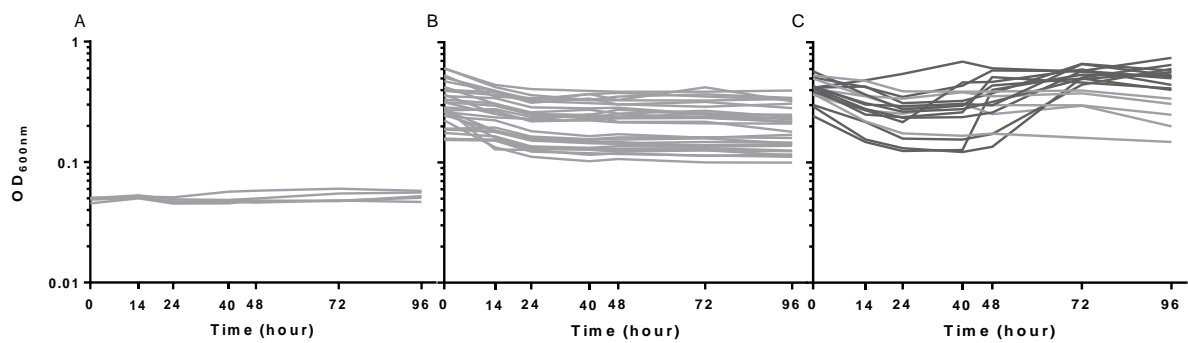
Supplementary Figure 7. Survival curves of DGRPs lines upon oral infection. Trajectories of survival of populations infected with *P. entomophila*, 5 dpi. Black lines are the control treatments: circles for the negative control and square for the positive control. Other curves correspond to different infected DGRP lines. Vertical bars correspond to standard error. Hazard ratios between survival percentages of flies of different DGRP lines. Positive control mortality curve functions as baseline. Vertical bars correspond to the standard error of the mean of the estimated hazard ratios. ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



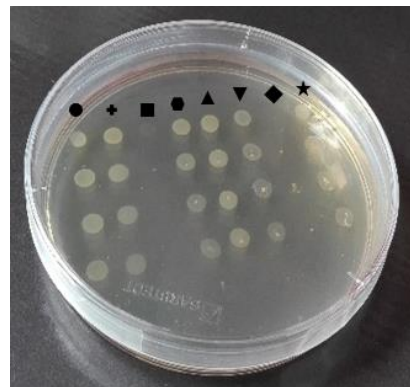
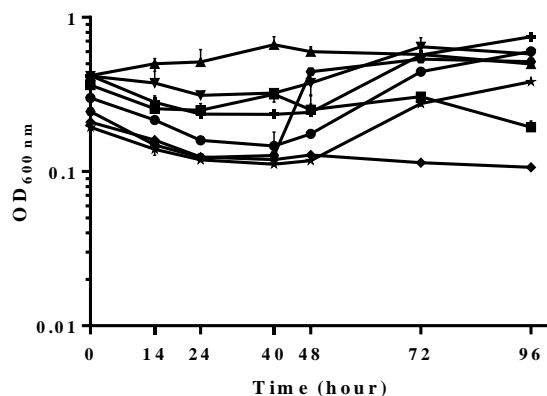
Supplementary Figure 8. Comparing data from the different studies. Comparison between the results obtained in the study by Bou Sleiman (2015)³⁵ and our project. The black bars correspond to the mortality percentage obtain by Bou Sleiman and the grey bars correspond to the mortality generated in our project. The strait horizontal black and grey bars correspond to the control mortalities, 70% and 50%, respectively. (A) Comparison of lines with mortalities below the controls. (B) Comparison of lines with mortalities above the controls. (C) Comparison of lines with contracting results.



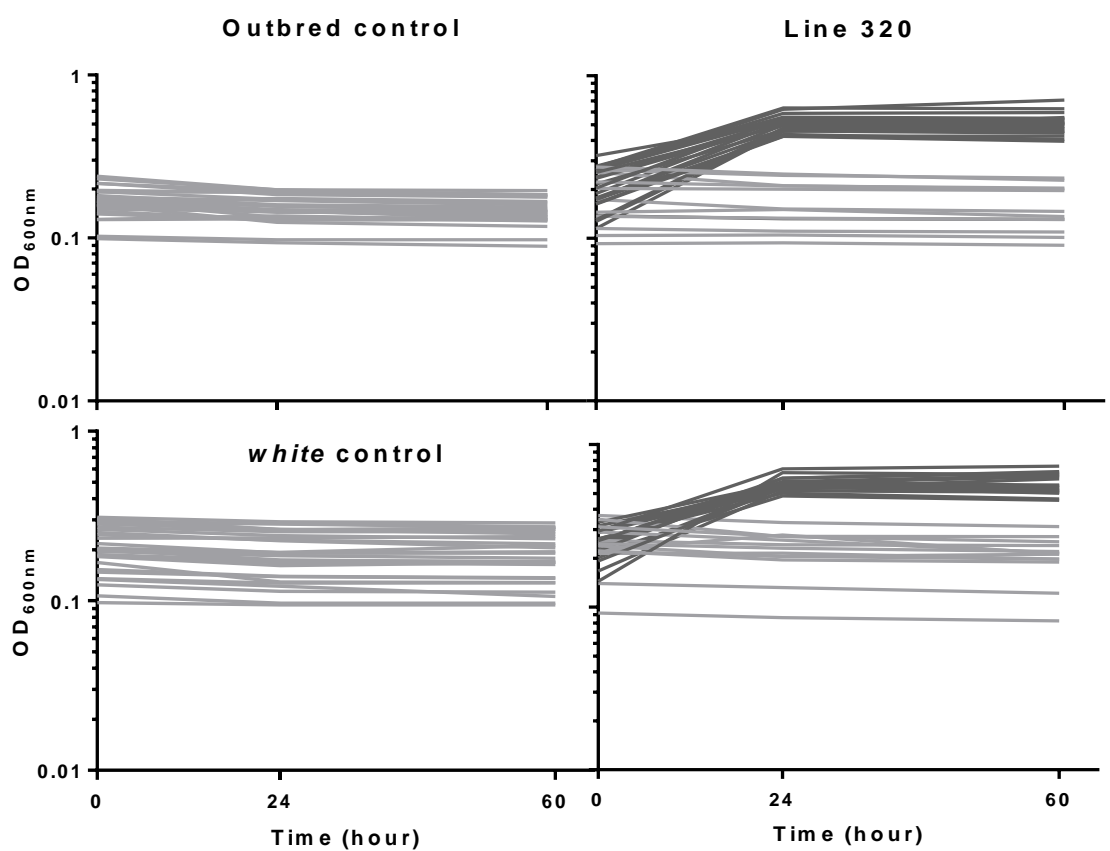
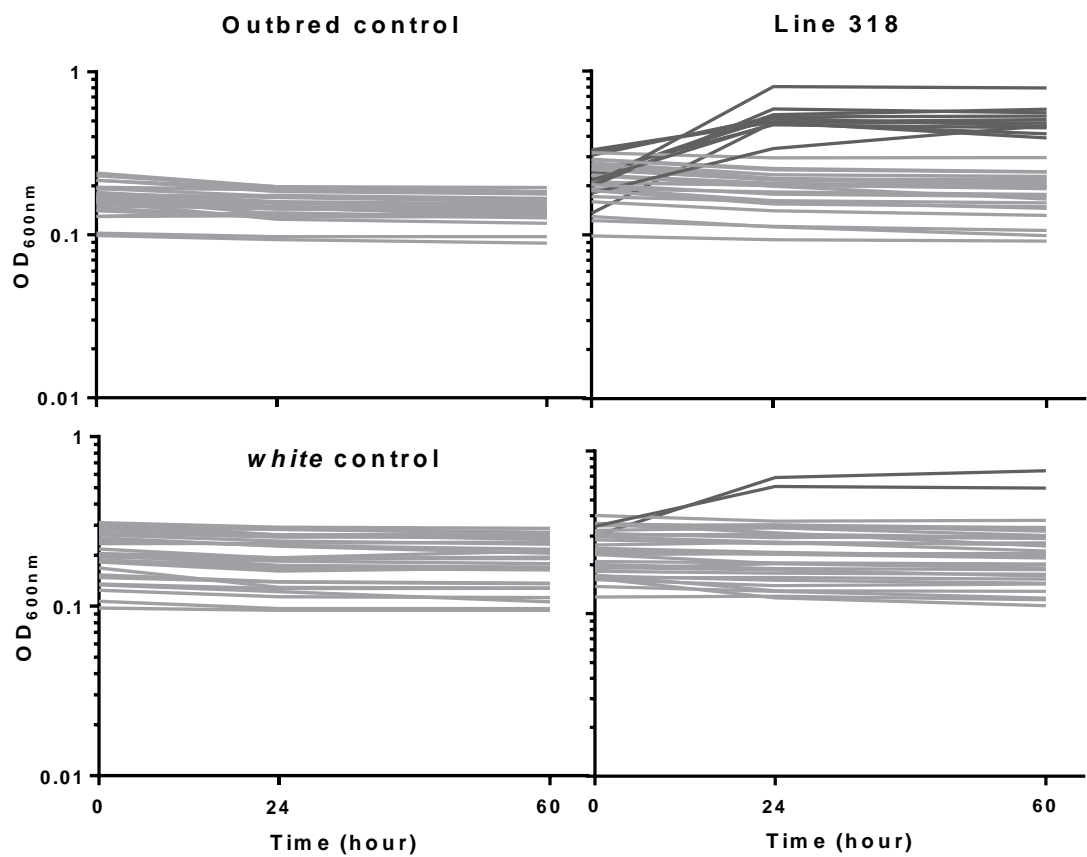
Supplementary Figure 9. Hazard ratios of all DGRP lines tested. Positive control mortality curve functions as baseline. Vertical bars correspond to the standard error of the mean of the estimated hazard ratios.

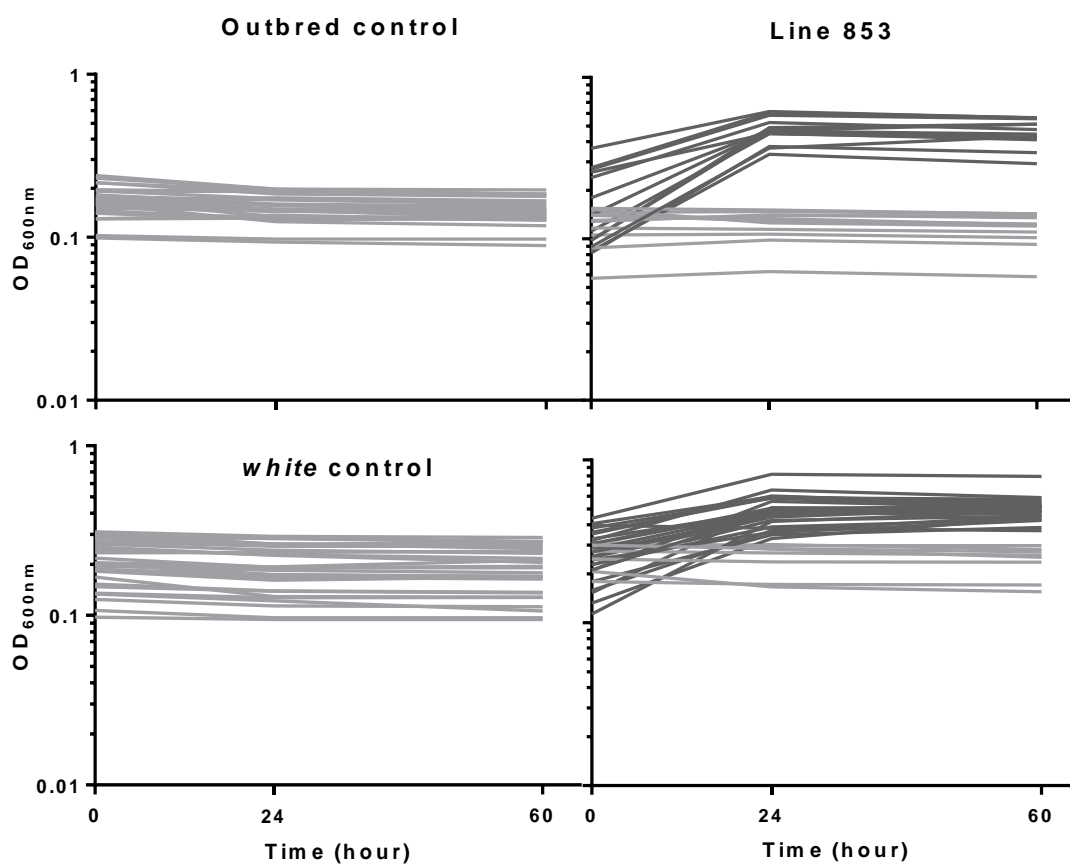
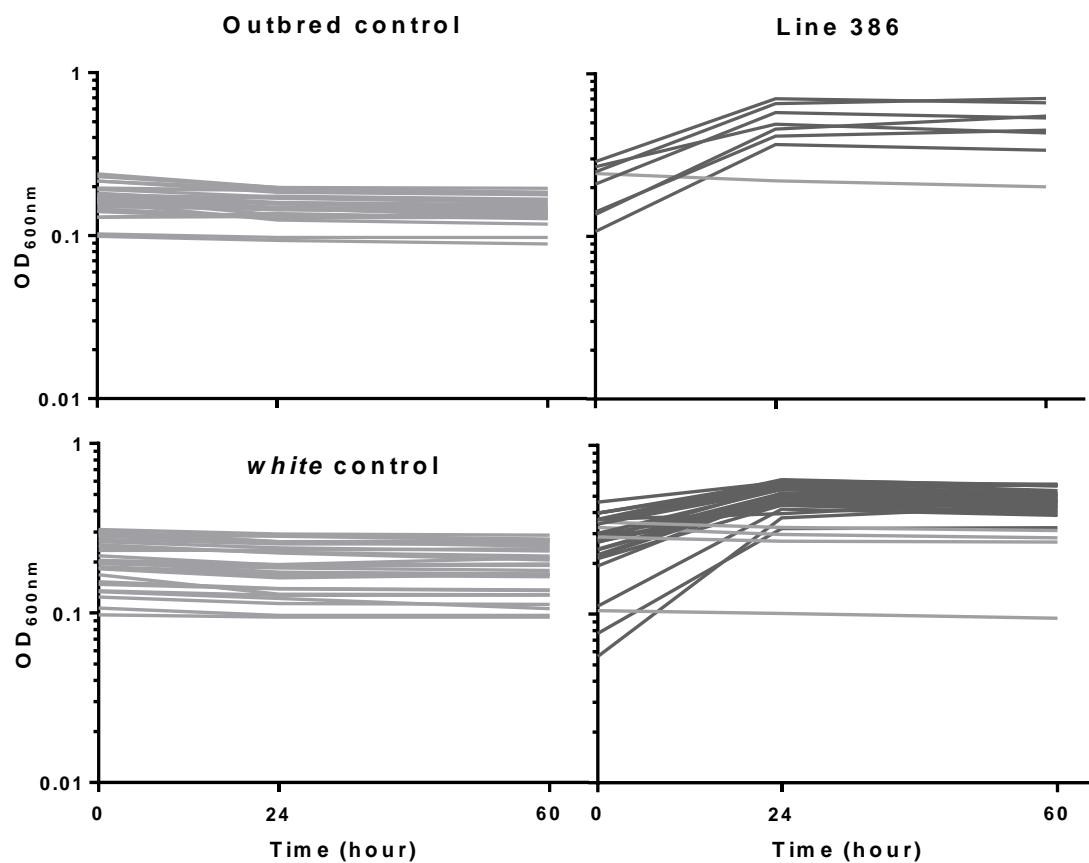


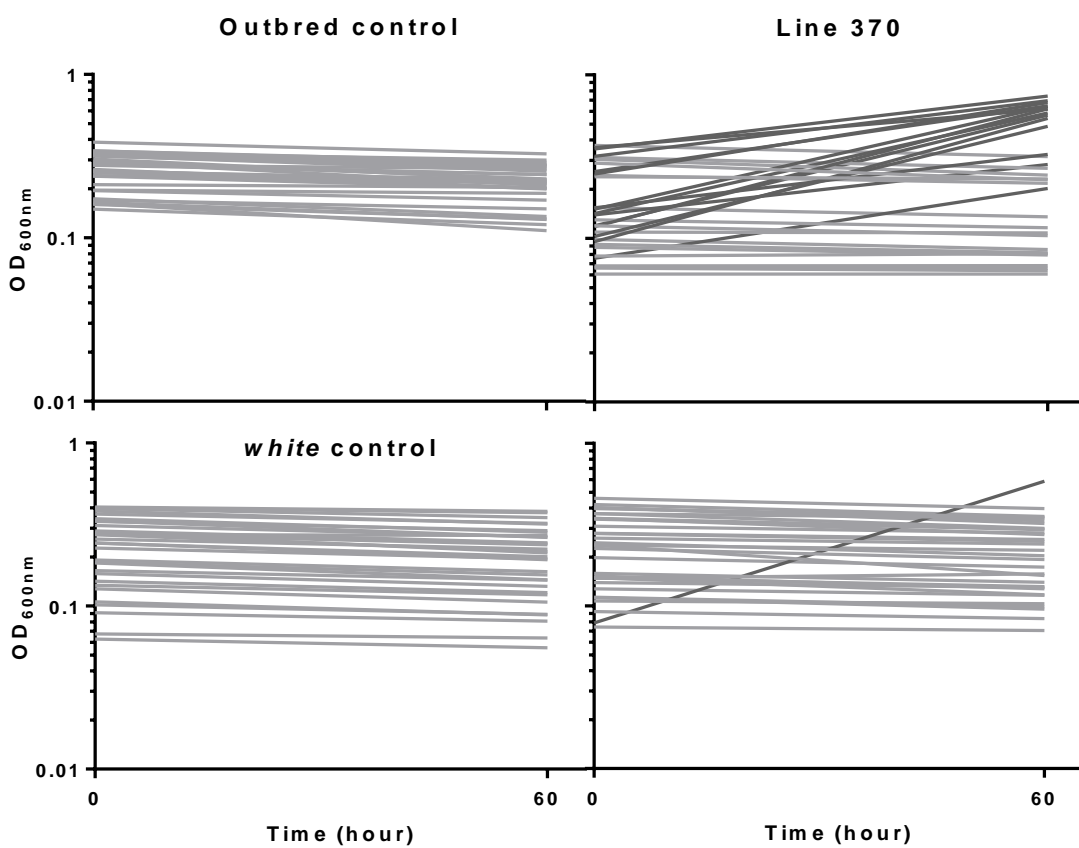
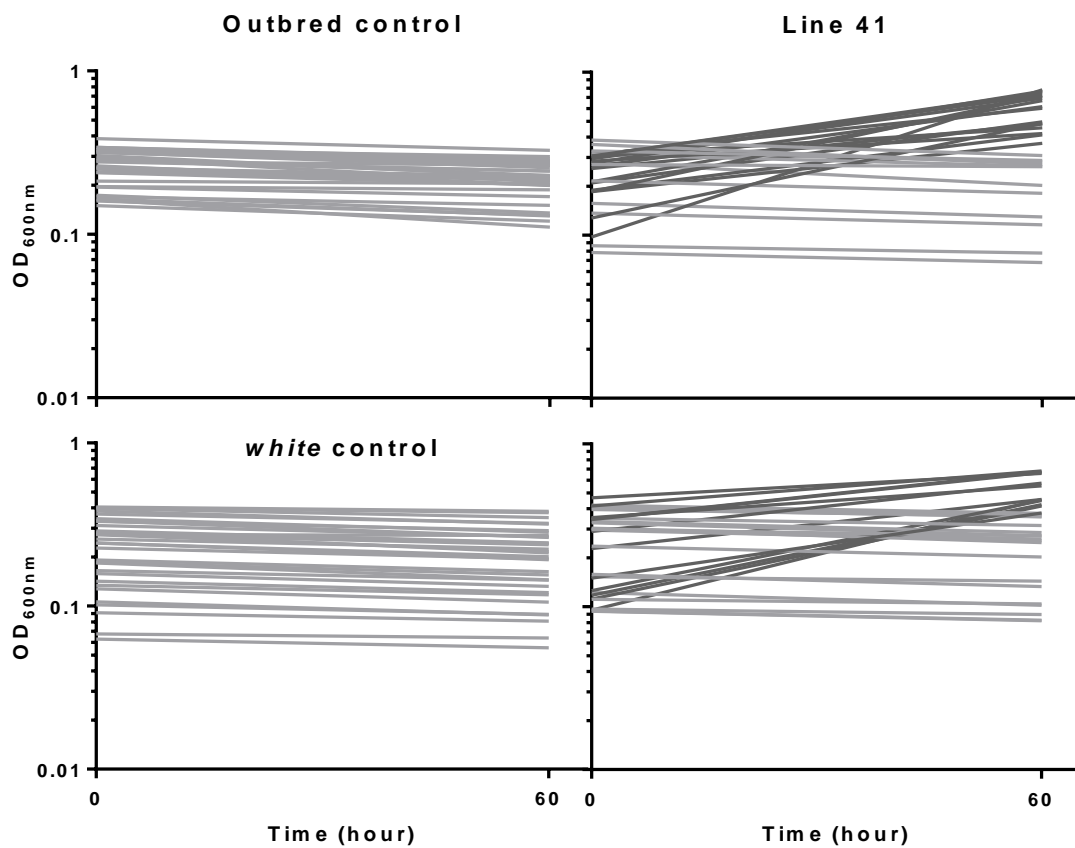
Supplementary Figure 10. Growth curves of infected flies measured in 96-well plates. Optical Density measurements through 96 hours in growth environment. For clarity, we separated the treatments: (A) OD of the base solution, LB+rifampicin (n=24); (B) OD of the non-infected control population (n=29); (C) OD of infected population (n=17); light grey, OD is stable or do not increase (5); dark grey, there is increase in the OD (12). Note that the Y axis is in Log10 scale and the X axis discriminates the time points at which optical density was measured.

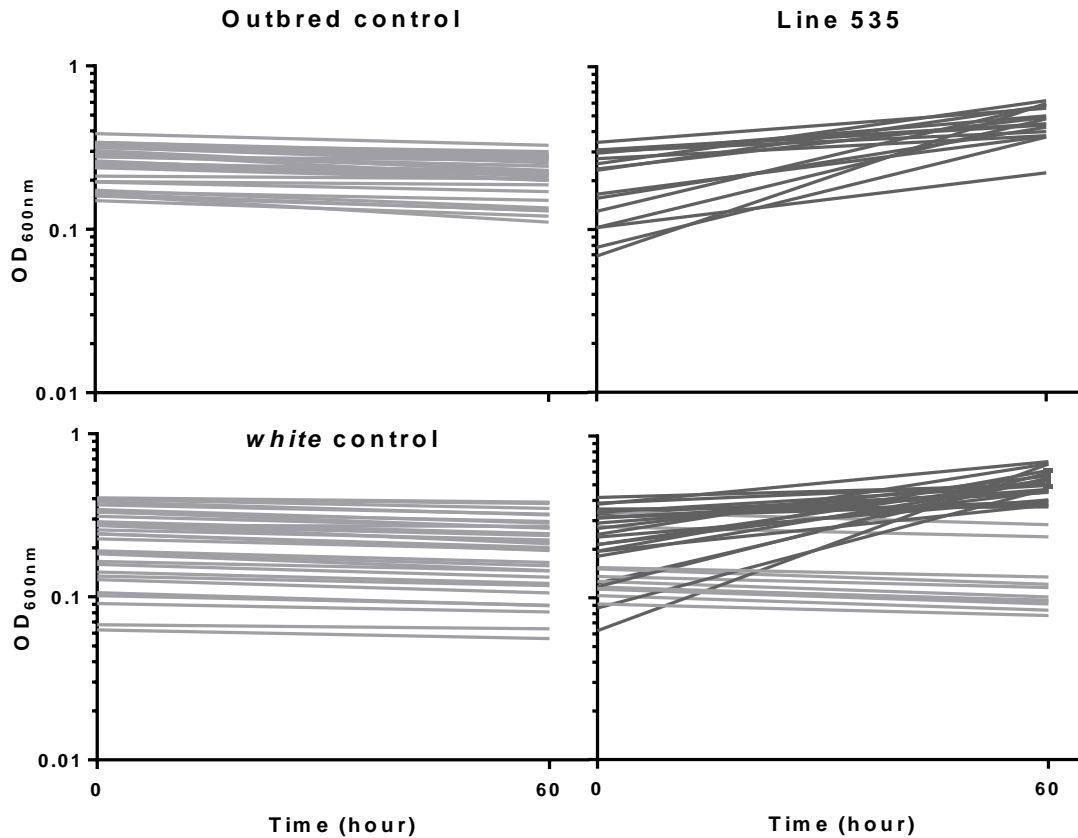


Supplementary Figure 11. Growth curves of infected flies measured in 96-well plates. Optical Density measurements through 96 hours in growth environment. Each column represents a curve and successive row, serial dilution. Each symbol represents the homogenate plating of respective curve.

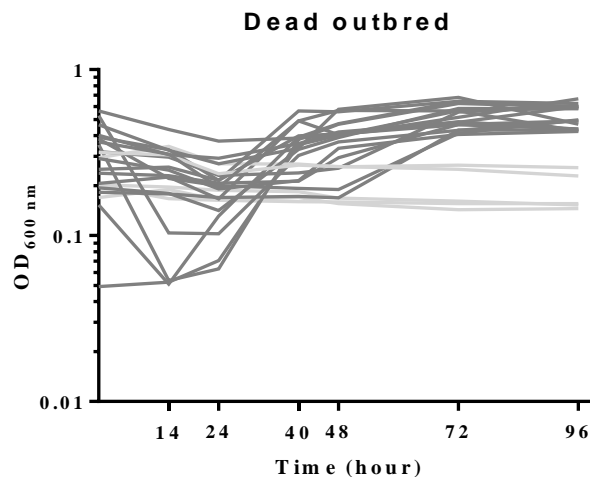








Supplementary Figure 12. Growth curves of infected flies measured in 96-well plates. Optical Density measurements through 96 hours in growth environment. For clarity, we separated the treatments: upper graphics represent the giver population (DGRP line) and below the receptor population. The graphs on the left represent the control flies of each population and the right the flies exposed to bacteria. The curves are divided in two groups: light grey, curves with stable or decreasing OD; dark grey, curves with increase in the OD. Note that the Y axis is in Log10 scale and the X axis discriminates the time points at which optical density was measured.



Supplementary Figure 13. Growth curves of dead flies measured in 96-well plates. Optical Density measurements through 96 hours in growth environment. The curves are divided in two groups: light grey, curves with stable or decreasing OD; dark grey, curves with increase in the OD. Note that the Y axis is in Log10 scale and the X axis discriminates the time points at which optical density was measured.

Supplementary Tables

Supplementary Table 1. See Figure 3.1. – Survival Curve, Previous (P) vs Adapt (A) protocol. Survival curve based on the Kaplan-Meier method.

Treatments	Test	Significance	<i>p</i> -value
Control; n=159 Infection; n=153	Log-rank (Mantel-Cox) test	****	<0.0001
Control; n=100 Infection; n=100		****	<0.0001

Supplementary Table 2. See Figure 3.2. – Survival curves under different feeding conditions. Survival curve based on the Kaplan Meier method.

Treatments	Test	Comparisons	Significance	<i>p</i> -value
Control; n=196 Cotton; n=202 Heat; n=201 Heat+yeast; n=219 Food; n=194 Food+yeast; n=194	Log-rank (Mantel-Cox) test	Control vs Cotton	****	<0.0001
		Control vs Heat	****	<0.0001
		Control vs Heat+yeast	*	0.032
		Control vs Food	****	<0.0001
		Control vs Food+yeast	**	0.0063
		Cotton vs Heat	****	<0.0001
		Cotton vs Heat+yeast	****	<0.0001
		Cotton vs Food	ns	0.8124
		Cotton vs Food+yeast	****	<0.0001
		Heat vs Heat+yeast	****	<0.0001
		Heat vs Food	****	<0.0001
		Heat vs Food+yeast	****	<0.0001
		Heat+yeast vs Food	****	<0.0001
		Heat+yeast vs Food+yeast	ns	0.4637
		Food vs Food+yeast	****	<0.0001

Supplementary Table 3. See Figure 3.3. – Quantity of bacteria in the filter paper discs through 24 hours. Non-Parametric test, Dunn's multiple comparison test. Comparison of mean rank between all columns. *P*-values were corrected for multiple comparisons.

Treatments	Test	Comparisons	Significance	<i>p</i> -value
1h; n=7 6h; n=7 24h; n=7	Multiple comparison test	1h vs 6h	ns	>0.9999
		1h vs 24h	*	0.0372
		6h vs 24h	*	0.0226

Supplementary Table 4. See Figure 3.4. – Food dye in the gut is a poor indicator of feeding. Parametric test, 2-samples t-test. Comparison of mean rank assuming the same standard deviation.

Treatments	Test	Significance	<i>p</i> -value
Color; n=20 Non-Color; n=20	2 sample t-test	ns	0.9028

Supplementary Table 5. See Figure 3.5. – Variation in bacteria load of infected flies. Parametric test, 2-samples t-test. Comparison of mean rank assuming the same standard deviation.

Treatments	Test	Significance	<i>p</i> -value
Control; n=10 Infected; n=40	2 sample t-test	****	<0.0001

Supplementary Table 6. See Figure 3.6. – Amount of bacteria outside infected flies. Parametric test, Holm-Sidak's multiple comparison test. Comparison of mean rank between all columns. P-values were corrected for multiple comparisons.

Treatments	Test	Comparisons	Significance	<i>p</i> -value
Control; n=10 Sterilized; n=20 Non- Sterilized; n=20	Multiple comparison test	Control vs Clean Control vs Non-Clean Clean vs Non-Clean	**** **** ns	<0.0001 <0.0001 0.7853

Supplementary Table 7. See Figure 3.7.A – Time-course in bacterial load over 72 hours and survival curve over 5 days. Parametric test, Holm-Sidak's multiple comparison test. Comparison of mean rank between all columns. *P*-values were corrected for multiple comparisons. Survival curve based on the Kaplan Meier method.

	Treatments	Test	Comparisons	Significance	<i>p</i> -value
A	Control; n=10 0h; n=20 24h; n=20 48h; n=20 72h; n=20	Multiple comparison test	Control vs 0h	****	<0.0001
			Control vs 24h	****	<0.0001
			Control vs 48h	*	0.0132
			Control vs 72h	ns	0.1532
			0h vs 24h	****	<0.0001
			0h vs 48h	****	<0.0001
			0h vs 72h	****	<0.0001
			24h vs 48h	*	0.0132
			24h vs 72h	****	<0.0001
			48h vs 72h	ns	0.1532
B	Control; n=203 Infection; n=200	Log-rank (Mantel-Cox) test	Control vs Infection	****	<0.0001

Supplementary Table 8. See Figure 3.8 – White outbred population survival upon *P.entomophila* oral infection. Survival curve based on the Kaplan Meier method.

	Treatments	Test	Comparisons	Significance	<i>p</i> -value	
A	Control Outbred; n=196 Infection Outbred; n=202 Control White; n=193 Infection White; n=178	Log-rank (Mantel-Cox) test	Outbred: Control vs Infection	****	<0.0001	
			White: Control vs Infected	****	<0.0001	
			Infection: Red vs White	***	0.0002	
B	Control Outbred; n=100 Infection Outbred; n=120 Control White; n=100 Infection White; n=100		Outbred: Control vs Infection	****	<0.0001	
			White: Control vs Infected	****	<0.0001	
			Infection: Red vs White	**	0.0030	

Supplementary Table 9. See Figure 3.9. – Horizontal transmission of *P. entomophila*. Survival curve based on the Kaplan Meier method. Shapiro – Wilk normality was performed to confirm the normality of the data.

	Treatments	Test	Comparisons	Significance	p-value
A	Control; n=214 Infection; n=220	Log-rank (Mantel-Cox) test	Control vs Infection	****	<0.0001
B	Wild type/donor; n=15 White/receptor; n=30	Shapiro-Wilk normality		****	<0.0001
		t-test		***	<0.0005

Supplementary Table 10. See Figure 3.10. – Trade-off hypothesis theoretical and experimental data. Point's distribution fitted with linear regression and second order polynomial curves.

Data		Test	Equation	R square
Virulence (%)	Transmission (%)	Linear regression	$y = 1.5517x + 8.882$	0.7847
0.500	3.125			
12.376	28.125			
20.745	62.500			
28.643	46.875	Second order polynomial	$y = -0.0329x^2 + 2.7918x + 2.3874$	0.8312
29.050	46.660			
38.000	66.670			

Supplementary Table 11. See Figure 3.12. – Contrasting survival analyses. Survival percentage of both studies.

Line	Bou Sleiman	From this study
320	4,3	17,98942
857	4,8	45,59586
142	9,7	11,41304
426	11,7	9,18367
217	15,0	55,61526
907	16,9	16,48936
332	20,7	6,25491
801	25,2	7,92079
373	40,8	29,53368
589	41,0	34,44444
513	42,7	29,58580
859	45,0	15,92039
41	46,1	17,96117
362	48,9	3,98010
177	52,7	94,17989
352	55,0	26,13065
508	57,3	30,32484
381	58,6	36,81592
324	68,2	40,09662
879	79,2	16,12904
138	93,4	10,08130
365	94,9	51,61290
595	96,0	86,17021
153	98,2	91,79487
176	100,0	97,79006

Supplementary Table 12. See Figure 3.13. – Measurements of virulence and transmission in DGRP lines. Point's distribution fitted with linear regression and second order polynomial curves.

Data			Test	Equation	R square
DGRP line	Virulence (%)	Transmission (%)	Linear regression	$y = 1.0391x$	0.3578
370	2.100	3.125			
320	4.348	59.375	Second order polynomial	$y = -0.0082x^2 + 1.6525x$	0.3949
41	8.490	38.710			
318	15.459	6.250	Third order polynomial	$y = 0.0004x^3 - 0.0588x^2 + 3.093x + 2.3874$	0.4643
535	57.143	60.600			
853	71.564	71.875			
386	86.800	87.500			

Supplementary Table 13. See Supplementary figure 3. - Survival curve, variability of the new protocol.

	Treatments	Test	Comparisons	Significance	p-value
A	Control; n=193 Replicate I; n=196 Replicate II; n=194 Replicate III; n=203	Log-rank (Mantel-Cox) test	Control vs RepI	****	<0.0001
			Control vs RepII	****	<0.0001
			Control vs RepIII	****	<0.0001
			RepI vs RepII	*	0.0160
			RepI vs RepIII	ns	0.0875
			RepII vs RepIII	ns	0.4822
B	Control; n=201 Replicate I; n=201 Replicate II; n=199		Control vs RepI	****	<0.0001
			Control vs RepII	****	<0.0001
			RepI vs RepII	ns	<0.9321
C	Control; n=193 Replicate I; n=178 Replicate II; n=185 Replicate III; n=181		Control vs RepI	****	<0.0001
			Control vs RepII	****	<0.0001
			Control vs RepIII	****	<0.0001
			RepI vs RepII	ns	0.1343
			RepI vs RepIII	ns	0.2892
			RepII vs RepIII	ns	0.6697
D	Control; n=193 Replicate I; n=198 Replicate II; n=197 Replicate III; n=198		Control vs RepI	****	<0.0001
			Control vs RepII	****	<0.0001
			Control vs RepIII	****	<0.0001
		RepI vs RepII	ns	0.8103	
		RepI vs RepIII	ns	0.2088	
		RepII vs RepIII	ns	0.1291	

Supplementary Table 14. See Supplementary figure 4. – Survival curve, using different bacteria quantities.

Treatments	Test	Comparisons	Significance	p-value
Control; n=205 OD25; n=202 OD50; n=202 OD75; n=188 OD110; n=199	Log-rank (Mantel-Cox) test	Control - vs OD25	ns	0.0958
		Control - vs OD50	****	<0.0001
		Control - vs OD75	****	<0.0001
		Control - vs OD110	****	<0.0001
		OD25 vs OD50	****	<0.0001
		OD25 vs OD75	****	<0.0001
		OD25 vs OD110	****	<0.0001
		OD50 vs OD75	**	0.0011
		OD50 vs OD110	****	<0.0001
		OD75 vs OD110	**	0.0080